liposomal targeting of glucocorticoids

a novel treatment approach for inflammatory disorders

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Liposomal targeting of glucocorticoids. A novel treatment approach for inflammatory disorders
Utrecht University, Faculty of Pharmaceutical Sciences, Faculty of Veterinary Medicine

ISBN 90-393-3285-1

Printed by PrintPartners Ipskamp B.V., Enschede, The Netherlands

Liposomal targeting of glucocorticoids

A novel treatment approach for inflammatory disorders

Liposomen als doelgerichte toedieningsvorm voor glucocorticoïden

Een nieuwe behandelingsstrategie voor inflammatoire aandoeningen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht

op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen,

ingevolge het besluit van het College voor Promoties,

in het openbaar te verdedigen op

vrijdag 14 februari 2003 des middags te 14.30 uur

door

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Geboren op 6 juli 1971 te Rotterdam

Promotores: Prof. dr. G. Storm
Prof. dr. D.J.A. Crommelin

Copromotor: Dr. M.H.M. Wauben

This research presented in Chapter 3, 4, 5, 7, 8, and 9 was part of the UNYPHAR project, a collaboration between the universities of Groningen, Utrecht and Leiden, and the pharmaceutical company Yamanouchi.

The research presented in Chapter 6 was financially supported by the Dutch Cancer Foundation.

The publication of this thesis was financially supported by:
Yamanouchi Europe B.V.
Faculty of Pharmaceutical Sciences, Utrecht University

‘The most beautiful experience we can have is the mysterious.
It is the fundamental emotion that stands at the cradle of true art and true science.
Whoever does not know it, who can no longer wonder and stand rapt in awe,
is as good as dead: his eyes are dimmed.’

Albert Einstein 1879-1955

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INTRODUCTION AND ORGANIZATION OF THE THESIS

1. Current status glucocorticoids in the treatment of inflammatory disorders

It was more than half a century ago when glucocorticoids were discovered as miraculously active compounds against inflammatory disorders, such as rheumatoid arthritis, asthma, allergic reactions, systemic lupus erythematosus and vasculitis. Their dramatic anti-inflammatory activity and broad applicability have made the introduction of glucocorticoids into the clinic one of the major breakthroughs of drug discovery research in the last century (1).

The enthusiasm about this new group of drugs gradually tempered when the toxicity profile became more apparent (2). A range of endocrinal, cardiovascular, musculoskeletal, dermatological and gastrointestinal effects was discovered strongly limiting the use of glucocorticoids in the clinic (3). Among the most pronounced side effects are osteoporosis (leading to fractures), skin atrophy, Cushing's syndrome, susceptibility for infections, hyperglycemia, ulceration of the stomach, psychiatric disorders, and hypertension (4-11). Due to the poor safety profile, systemic (i.e. oral, intravenous and intramuscular) treatment with glucocorticoids became only reserved for severe, refractive forms of inflammatory diseases in which other drugs were not sufficiently active.

The finding that the detrimental musculoskeletal and dermatological side effects of glucocorticoids were more related to long-term use of glucocorticoids offered some perspective (12). The disease course of inflammatory disorders is often characterized by exacerbations (relapses) of inflammation besides the long-term progression of tissue damage (13,14). Short-term treatment with repeated injections of high doses of glucocorticoids is one of the few strategies that can rapidly suppress exacerbations. This so-called 'pulse therapy' involves three to five (alternate) daily i.v. injections of up to 1 gram of (methyl)prednisolone or 0.1-0.2 g dexamethasone. Although cardiovascular, endocrinal and gastrointestinal side effects still do occur, pulse therapy is generally fairly well tolerated (15).

Topical administration proves to be the primary strategy to avoid (systemic) adverse effects. Application on the skin proved to be successful in treatment of inflammatory skin disorders while inhalation of glucocorticoids became the first choice treatment of asthma and allergic rhinitis (16). However, local treatment is only attractive when the target organ is easily accessible, which is often not the case. In rheumatoid arthritis local treatment can be realized by means of intra-articular injection (5-10 mg methylprednisolone acetate or 5 mg triamcinolone acetonide are most commonly used). However, intra-articular treatment requires a special technique and to prevent local reactions such as osteonecrosis, a particular joint should not be given more than 4-5 injections a year. Furthermore, this treatment approach is only attractive when single large joints are affected. In most cases rheumatoid arthritis involves a range of (smaller) affected joints and systemic therapy remains the only option (17).

Besides the poor safety profile, also the poor pharmacokinetic behavior limits the usefulness of glucocorticoids in systemic therapy (18). Glucocorticoids are drugs with a relatively high clearance rate and a large volume of distribution. This implies that to reach pharmacologically active drug levels at the site of inflammation, high and frequent doses

must be administered. The majority of these systemically administered doses localizes in healthy non-target tissues if not rapidly excreted from the body. To increase the amount of drug at the target site after systemic administration and to decrease localization at non-target tissues the so-called 'drug targeting' approach may offer perspective. Drug targeting makes use of colloidal carrier systems in which the drug is incorporated or to which the drug is attached. Distribution of the carrier-associated drug to organs/tissues is reduced, as the carrier cannot diffuse into extravascular tissues.

2. Drug targeting to inflamed sites

Drug targeting can be defined as a treatment approach leading to increased localization of a therapeutic agent at the target site in the body. Drug targeting can be realized by employing a drug carrier, which shows preferential affinity for the (pathological) target tissues. The increased target concentration of the carrier-associated drug as compared to the free drug may lead to a higher activity of the drug at the target site and/or reduced side effects at healthy non-target tissues.

Drug targeting may be considered as a means of local administration that makes use of the systemic route, since ideally drug targeting leads to 100% delivery of the systemically administered drug at the target site. Approaching this ideal situation depends on the type of drug carrier and target site. For instance, drug targeting to organs in which the mononuclear phagocyte system (MPS) is present (such as liver, spleen and bone marrow) can be highly efficient, as phagocytes in these organs are exposed to the blood circulation and rapidly take up the majority of the injected drug carrier (19). Meanwhile this phenomenon has compromised the use of drug carriers for targeting to non-MPS sites and additional modifications were necessary to enable significant drug delivery to these sites.

Over the last decades, a range of carriers has been investigated for the purpose of drug targeting. These include: plasma proteins, soluble antibodies, lipoproteins, nanoparticles, viruses, erythrocytes and erythrocyte-based structures, blood platelets, polymers, polymeric micelles and liposomes (20-31). To impose target selectivity to these carriers targeting ligands can be attached, such as antibodies or natural ligands for target site-specific receptors. This approach is often referred to as 'active drug targeting'. Some of these carriers may show target selectivity without the use of targeting ligands. The above-mentioned affinity of several unmodified drug carriers for the mononuclear phagocyte system is just an example. This phenomenon is usually referred to 'passive drug targeting'.

Interestingly, the approach of passive drug targeting can effectively be employed in inflammatory diseases. Inflammation generally results in locally increased vascular permeability, enabling cellular, (macro)molecular and colloidal blood components to extravasate and enter the inflamed site. I.v. injected colloidal drug carriers that stay long enough in the circulation spontaneously extravasate into sites of inflammation as well. This phenomenon of preferential localization at inflamed areas has been shown to occur with several colloidal drug carriers (32-34). Of these colloidal drug carriers, so-called 'long-circulating liposomes' may be among the most attractive ones.

3. Long-circulating liposomes

Liposomes are small lipid bilayer vesicles enclosing an aqueous core in which water-soluble drugs can be entrapped. The attractiveness of liposomes as drug carrier relates to their relatively high drug loading capacity, good biocompatibility, low toxicity, versatility and ease of preparation. Creating a lipid film by evaporation of a solution of membrane-forming lipids and subsequently hydrating this film with the aqueous drug solution spontaneously results in the formation of drug-encapsulating liposomes. Repeated extrusion, sonication or high-shear homogenization can be employed to produce liposomes of the desired size. The unencapsulated drug can easily be removed by dialysis, gel-filtration or centrifugation (35). Size and lipid composition are crucial parameters determining the *in vivo* behavior of liposomes after systemic administration. First, to prevent leakage of the drug from liposomes both upon storage and after injection in the circulation, stability of the drug-containing liposome formulation must be investigated and optimized. Second, the liposomes must be sufficiently small and have a sufficiently long circulation half-life so that they are allowed to extravasate at sites of pathology to a significant extent. Liposome formulations that meet these requirements/specifications are generally referred to as 'long-circulating liposomes'' (31).

The most extensively studied long-circulating liposome systems are PEG-liposomes. This liposome type is created by grafting low-molecular poly(ethylene glycol) (PEG) to the lipid bilayer surface (Figure 1). It is generally thought that surface-grafted water-soluble polymers can oppose adhesion of plasma proteins to the liposomes that would otherwise 'tag' liposome for recognition and subsequent premature uptake by phagocytes of the mononuclear phagocyte system. PEG-liposomes can circulate with a half-life as long as 24 hrs in rats and up to 50 hours in humans. PEG-liposomes have been developed for the targeted delivery of cytotoxic agents to tumors, as the tumor vasculature is often loosely organized, providing increased access for blood components (36). Several liposomally encapsulated cytotoxic agents are on the market or in late phase clinical trials (37,38).

Preclinical and clinical studies have revealed that i.v. administered PEG-liposomes can indeed preferentially localize in inflamed tissues. For instance, Brouwers et al. showed that PEG-liposomes can be employed for the scintigraphic detection of inflamed sites in patients with Crohn's disease (39). Despite these results, PEG-liposomes have hardly been used for the purpose of drug targeting in inflammatory disorders. Corvo et al. reported increased therapeutic activity of superoxide dismutase (SOD) upon encapsulation in PEG-liposomes and i.v. administration in an experimental rat model of arthritis (40). Disappointing results were obtained with the disease-modifying anti-arthritic drug methotrexate in PEG-liposomes (41). Although encapsulation of glucocorticoids in long-circulating liposomes for targeted delivery in inflammatory disorders seems logic and straightforward, the approach has never been proposed and investigated. Only a few studies on liposomal formulations of glucocorticoids for local, intra-articular treatment of inflamed joints in experimental arthritis exist (42,43).

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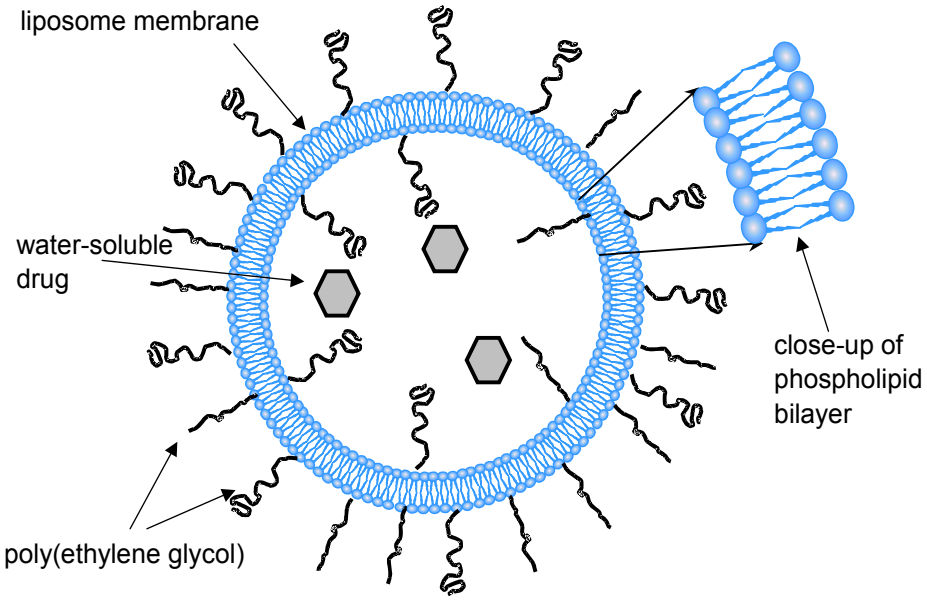

Figure 1. Schematic representation of a drug-containing long-circulating PEG-liposome

4. Aims and outline of this thesis

The primary aim of this thesis was to investigate application of PEG-liposomes as passive targeting system for the delivery of glucocorticoids to sites of inflammation. Chapter 2 provides a background to the field of advanced drug delivery with liposomes. The present status of both passive and active drug targeting with liposomes is reviewed.

Chapter 3 describes the pharmacokinetics, tissue distribution, degree of inflamed joint targeting and therapeutic activity of prednisolone phosphate-PEG-liposomes in rat adjuvant arthritis, a widely accepted animal model for rheumatoid arthritis. Chapter 4 also addresses the anti-arthritic effect of prednisolone phosphate-PEG-liposomes, but in a different model: murine collagen induced arthritis. This model allows a mechanistic study of the localization and anti-arthritic effect of liposomal glucocorticoid at a microscopic level. Besides application for arthritis treatment, we also studied the therapeutic activity of liposomal glucocorticoid in experimental models of multiple sclerosis. Chapter 5 describes pharmacokinetics, the degree of targeting to the inflamed nervous system and the beneficial effects of prednisolone phosphate-PEG-liposomes, both at a microscopic as well as a macroscopic level, in comparison to standard treatment with (free) methylprednisolone. To our surprise, liposomal prednisolone phosphate not only proved to be highly effective in models of inflammatory disorders, but also in murine tumor models, as reported in Chapter 6. The effect on tumor growth is evaluated both macroscopically as well as under the microscope. Some possible mechanisms are discussed that could explain this unexpected antitumor effect.

Chapter 7 describes the evaluation of glucocorticoids other than prednisolone in PEG-liposomes in experimental arthritis. Dexamethasone and budesonide are studied regarding both therapeutic activity and systemic adverse effects. An approach is proposed and evaluated to optimize the therapeutic index of liposomal glucocorticoid by encapsulating inhaled high-clearance glucocorticoids. Chapter 8 addresses the phenomenon of complement-related hypersensitivity reactions, which has been observed with PEG-liposomes in the clinic. The study in this chapter aims at finding the responsible key factor(s) and proposes a long-circulating liposome type that does not induce complement activation. Since the biological fate of liposomes-attached PEG after cellular uptake is not known, it was the objective of the work presented in Chapter 9 to design a biodegradable polymer-lipid conjugate for the preparation of long-circulating liposomes. Targeted delivery of glucocorticoids and therapeutic activity in experimental arthritis of this new type of liposome is also evaluated. Finally, Chapter 10 provides a summary and a general discussion of the results presented in this thesis.

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CHAPTER 2

LIPOSOMES FOR INTRAVENOUS DRUG TARGETING: DESIGN AND APPLICATIONS

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ABSTRACT

Drug targeting with liposomes has been studied for over 25 years and has demonstrated its value in clinical practice. This minireview offers an overview of the design and application of liposomes for i.v. drug targeting. Two approaches are outlined: passive and active targeting. The former approach is based on liposomes with prolonged circulation and selective target localization properties, while in the latter approach specific targeting ligands are coupled to the liposome surface in order to achieve enhanced interaction with target cell membranes.

I. INTRODUCTION

Successful treatment of life-threatening and chronic diseases by intravenous (i.v.) administration of therapeutic agents often involves relatively high and frequent dosing. Due to rapid elimination or a large volume of distribution, many drugs poorly accumulate at target sites while large amounts are wasted or unintendedly localize at healthy tissue sites. As a consequence, a systemic treatment approach is frequently limited by toxicity and therefore characterized by a low benefit/risk ratio. For decades research has been focusing on the possibility of encapsulating drugs in carrier vehicles that take their drug load specifically to target sites in the body, meanwhile protecting it against rapid degradation and/or elimination and preventing undesired localization in non-diseased organs ('drug targeting').

Among a variety of drug carrier systems, liposomes (small, biocompatible lipid-bilayer vesicles, see Figure 1) have been investigated extensively and the preclinical and clinical findings have demonstrated their versatility to accommodate a large variety of drugs for a wide range of therapies (1,2). The attraction of liposomes as drug carrier system was initially based on expectations of good biocompatibility, low toxicity and a lack of immune system activation or suppression. These assumptions were based on the fact that liposomes are typically composed of natural lipids that form bilayers with structural resemblance to cell membranes. Although reality turned out to be more complex, the approval of several liposome-based pharmaceutical products in the last decade illustrates a growing acceptance of the liposomal delivery system as an important parenteral drug formulation.

A breakthrough in the liposome research field has been the finding that i.v. injected liposomes have the ability to spontaneously localize into sites of pathology ('passive targeting') (3,4). It was this finding that facilitated clinical development of liposomes for therapeutic purposes. In certain cases however, it remains desirable to couple a specific targeting ligand to the surface of the liposomes to achieve receptor-mediated target cell binding ('active targeting') (3). This minireview aims at providing the reader with a condensed overview of the design and application of liposomes for i.v. drug targeting. Both the passive and active strategy will be discussed.

II. PASSIVE TARGETING

One of the important barriers limiting the application of liposomes for intravenous drug targeting has been a short blood circulation time resulting from rapid and efficient recognition and removal from blood by cells of the mononuclear phagocyte system (MPS), particularly those in the liver and spleen. This immune system's first line of defense consists of macrophages specialized in nonspecific elimination (phagocytosis) of all exogenous material in the circulation, including liposome particles. Plasma proteins like antibodies and other so-called opsonins recognize and adhere to liposomal bilayers, provoking the uptake of liposomes by MPS-macrophages (5,6). This MPS-directed behavior of liposomes has been successfully exploited to achieve selective delivery of antimicrobials in models of intracellular infections caused by pathogens localized in MPS cells (7). However, in the majority of diseases, the rapid sequestration by the MPS often eliminates the intended beneficial effects and moreover can pose considerable risk of toxicity to these cells (8,9).

Therefore, the introduction of liposomes exhibiting prolonged circulation by virtue of their capability to oppose rapid MPS uptake represents a milestone in liposomal drug delivery research. These newer forms of liposomes (referred to as long-circulating liposomes (LCL)) are actively being investigated worldwide and the results have substantially expanded the role of liposomes in developing new therapeutics (see Table 2). The key factor responsible for the increased interest in liposome drug delivery is the observation that LCL spontaneously and selectively accumulate at sites of enhanced vascular permeability that are fortunately present in diseased tissues like tumors and areas of infection and inflammation (3,4). This phenomenon is usually referred to as 'passive targeting'. The explanation for the fascinating passive targeting effect is straightforward: since LCL are generally smaller than the 'pores' that appear in the endothelial linings at pathological sites, their prolonged circulation property increases the chance that they extravasate into the extravascular space. Retention of LCL at these sites will lead to accumulation and the creation of a relatively high local drug concentration (10).

The ability to passively target drugs to extravascular sites of pathology via LCL is dependent on a combination of:

1. Prolonged blood circulation, providing ample opportunities to encounter the region of disease.
2. Adequate access to the pathological tissue and target cells therein.
3. Ability of the LCL to interact with target cells and to deliver the encapsulated drug in an active form.

Each aspect is briefly addressed below.

II.1 Prolonged circulation

Qualitatively, the mechanism behind the approaches taken to enhance the residence time of liposomes in the blood compartment is generally explained by reduction of the adsorption of various blood components onto the liposomal surfaces (e.g., adsorption of proteins

interacting with one or more receptors on the MPS-macrophage cell surface, a process termed opsonization). Thus liposomes types able to resist rapid opsonization are likely to show prolonged blood circulation times (11,12).

One of the first major advances in prolonging blood residence was made possible through careful studies of the dependence of MPS-uptake on liposomal lipid composition (13). These studies led to findings that small (i.e., less than about 100 nm in diameter), neutral and rigid (i.e., composed of fully saturated lipids and a high cholesterol content) liposomes can exhibit prolonged circulation, but only at relatively high lipid doses (13,14). This success in generating LCL has been exploited for development of the marketed liposomal formulations of the anticancer drug daunorubicin and the antifungal drug amphotericin B (15,16). (Table 2). It is believed that the use of highly cohesive bilayers inhibits interaction of plasma proteins with the liposome particles. Consequently, the opsonic proteins are not able to induce the surface modifications which otherwise would 'mark' the liposomes for MPS uptake. Another approach to create LCL utilized the inclusion of specific glycolipids such as monosialoganglioside Gm1 or phosphatidylinositol. It was hypothesized that these glycolipids act through creating a carbohydrate 'shield' over negatively charged groups located underneath (17). Overall, these methods achieved some success, but are all dependent on rigid liposome bilayers, which can impose a limitation when fluid bilayers are needed to achieve appropriate drug release rate profiles *in vivo*.

A more recent development to prepare LCL with less restriction to lipid bilayer composition is based on modification of the liposome surface with hydrophilic polymers to protect the lipid surface of the liposome against protein adsorption and consequent uptake by mononuclear phagocytes. A list of polymer coatings investigated over the years is presented in Table 1. Stable coating with hydrophilic polymers is generally achieved by coupling the polymers to lipid anchor molecules that can insert into the liposome bilayer. The hydrophilic part of the conjugate is believed to form a repulsive steric barrier that can 'hide' the liposome bilayer from plasma proteins (18-20).

At present, by far the most extensively explored coating polymer is polyethylene glycol (PEG). The PEGylation strategy is often referred to as 'steric stabilization' or 'Stealth technology'. Typically an incorporated molar amount of 5% proves to be sufficient to achieve prolonged circulation (25). In rats the plasma half-life of a 100 nm PEG-coated liposome is around 20 – 24 hrs while in humans a half-life of 45 hrs can be realized (26). PEG surface modification has been shown to have important advantages over the other methods to obtain prolonged circulation behavior (27). One important claimed advantage is that PEG-liposomes possess, within certain limits, dose-independent log-linear blood concentration-time profiles (28). This permits dose escalation without complications arising from changes in pharmacokinetic behavior. Another advantage is the possibility of varying the lipid composition without affecting circulation time and tissue distribution, which provides an ability to optimize the liposome physicochemical properties for drug loading and release (29).

Table 1. Polymers with capacity to extend the circulation time of liposomes

Polymer	Reference
Poly(ethylene glycol)	(4)
Poly(acrylamide)	(21)
Poly(vinyl pyrrolidon)	(21)
Poly(acryloyl morpholine)	(22)
Poly(2-methyl-2-oxazoline) and Poly((2-ethyl-2-oxazoline)	(23)
Poly(vinyl alcohol)	(24)
Hydroxypropylmethylcellulose	(24)

II.2 Localization at pathological sites

Besides rapid uptake by MPS-macrophages, another significant barrier for i.v.-injected particulate systems is the endothelial lining between the vascular space and extravascular target tissue. In most tissues the vascular system is lined with a continuous layer of endothelial cells often supported by a basement membrane. This barrier virtually excludes extrasavasation of particles such as LCL except for a few selected sites where the endothelial lining is discontinuous. Fortunately, it has been found that regions of increased capillary permeability include pathological sites such as tumors and sites of infection and inflammation. LCL have been shown to extravasate into these pathological areas (3,4). The mechanism(s) of extravasation (often referred to as 'enhanced permeability and retention (EPR) effect') is not well understood but can be thought of simply as 'leakage in the plumbing' (10). Inflammatory processes are accompanied by locally increased vascular permeability. In case of tumors, the angiogenesis process results in tumor blood vessels with increased permeability. Up to 10% of the injected LCL dose has been shown to localize in such sites of pathology, suggesting potential for substantial improvements in efficacy of encapsulated therapeutic agents that are active towards these pathologies.

II.3 Therapeutic availability

Conceptually, many agents could benefit from enhanced delivery to the pathological target and/or reduced distribution to healthy tissues. At present abundant literature is available showing that many therapeutic agents indeed profit from encapsulation in LCL by passive targeting resulting in enhanced localization in diseased tissues (Table 1). These results suggest broad applicability of LCL for drug delivery. For most LCL-entrapped drugs improved efficacy requires a liposomal composition capable of retaining the drug in the LCL during prolonged circulation but releasing it once the LCL have accumulated in the site of pathology, the latter aspect being referred to as therapeutic availability. These requirements have apparently been met by several formulations, as they show striking therapeutic activities in animal disease models and in some cases in humans.

As LCL are designed to be stable in the circulation, release of the active ingredients at the target site cannot be taken for granted (see also Figure 2, option 1). If the LCL remain intact at the target site, the release of drug will be a time-consuming process and local therapeutic levels of active drug are only slowly achieved. Fortunately, at sites of inflammation and tumors enzymes are active that can cause degradation of the liposome phospholipid bilayer (30). Especially LCL without polymer coating are expected to be affected by enzymatic degradation. A hydrophilic polymer coating may hamper the degradation process, although recently, it has been shown that inclusion of PEG in the lipid bilayers appears to enhance enzymatic degradation (31).

When enzymatic degradation at the target site is insufficient, drug release may be achieved with triggering by external means. For over two decades researchers have been investigating the possibility of using thermosensitive liposomes. This approach is based on release of drug from the liposome as a result of enhanced fluidity of normally rigid liposome bilayers, when target tissue is heated above the transition temperature of the lipid composition. Several studies show that this concept can indeed result in increased therapeutic efficacy of a liposomally encapsulated drug (32,33). Another potential mechanism for drug release within the target site is uptake and intracellular processing of drug-LCL by local phagocytes. Intracellular degradation of the LCL bilayers may liberate the drug, which may subsequently diffuse out of the endosomal compartment and become active in the cytoplasm of he phagocyte. Successful targeting and modulation of macrophage populations at inflamed tissue has been achieved with long-circulating liposomes containing clodronate that can cause cell death when it becomes intracellularly available (34,35). Additionally, when a drug is able to pass membranes, it may eventually diffuse out of the phagocyte into the extracellular environment and thereby become available for interaction with the intended target cells. This concept of ‘macrophage-mediated drug release’ has been exemplified with the antitumor drug doxorubicin (36).

It should be mentioned that drug release within the target site is not always required. This is the case when the LCL are used for diagnostic purposes. LCL-based formulations containing isotopes for scintigraphic imaging of infection and inflammation have been shown to represent promising radiopharmaceuticals in nuclear medicine (37,38). Table 2 shows the developmental status of the various liposomal pharmaceuticals that are currently in clinical and preclinical studies.

Table 2. Current developmental status of intravenous liposomal therapeutic agents

Status	Active ingredient (product name)	Therapeutic field	Reference
Commercially available	Doxorubicin (Doxil/Caelyx™)	Oncology	(39,15)
	Daunorubicin (Daunoxome™)		(39,15)
	Amphotericin (Ambisome™)		(40,41)
Clinical study Phase III	Tretinoin (Atragen™)	Oncology	(42)
	Nystatin (Nyotran™)	Fungal infections	(43)
	Amikacin (Mikasome™)	Fungal infections	(44)
	Prostaglandin E1 (Ventus™)	Acute Respiratory Distress Syndrome	(45)
Phase II	Cisplatin (SPI-77)	Oncology	(46)
	NDDP, a cisplatin analogue		(47)
	Vincristine (ONCO-TCS)		(48)
Phase I	Annamycin	Oncology	(49)
	Paclitaxel		(50)
Preclinical, animal study	Gentamicin	Bacterial infections	(51)
	Ceftazidime		(52)
	Streptomycin		(53)
	Kanamycin		(54)
	Cefoxitin		(55)
	Clofazimine		(56)
	Rifampicin		(57)
	Isoniazid		(57)
	Irinotecan (CPT-11)	Oncology	(58)
	Boron derivatives for Boron Neutron Capture Therapy		(59,60)
	GL147211C, camptothecin analogue		(61)
	TNF-alpha		(62)
	Adriamycin		(63)
	Interleukin-2		(64)
	Epirubicin		(65)
	MTP-PE, muramyl tripeptide derivative		(66)
	CNDAC, beta-D-arabino-furanosylcytosine derivative		(67)
	20(S)-Camptothecin	Leishmaniasis	(68)
	Amarogentin		(69)
	Atovaquone		(70)
	N-methylglucamine antimoniate		(71)
	Superoxide Dismutase	Arthritis	(72)
	Metotrexate		(73)
	Indomethacin		(74)
	Clodronate		(75)
	Cyclosporin	Transplantation	(76)
	Busulphan		(77)
	Hemoglobin	Blood substitute	(78)
	Albuterol	Inflamed airways	(79)

Taking together, passive targeting and improved therapeutic behavior of LCL-encapsulated drugs is based on selective but non-specific extravasation into pathological tissues accessible from the circulation due to a locally increased vascular permeability. In most cases mentioned in Table 2, drug release takes place extracellularly. Subsequently, the released drug is able by itself to reach the therapeutic intervention site (often intracellular), which often will require passive diffusion over membrane barriers. However, when target cells are not localized in the extravascular space but for example in the blood circulation, the localization process requires more sophisticated strategies such as specific carrier-target cell recognition. In addition, an increasing number of new drug molecules, especially the new biotechnology derived agents such as proteins and nucleic acids, can not readily pass cell membranes due to their hydrophilicity and relatively high molecular weight. These molecules require liposomal carriers that are able to deliver the entrapped drug to the subcellular target compartment (often the cytoplasm or nucleus). In these situations surface-conjugated targeting ligands and/or membrane-translocating functionalities for intracellular delivery have to be included in the LCL system. Both ligand-mediated active targeting of liposomes and new approaches to obtain cytosolic drug delivery are discussed in more detail below.

III. ACTIVE TARGETING

Active targeting of liposomes refers to the conjugation of site-directing ligands to the surface of liposomes to obtain specific binding to cell receptors on the surface of the target cells. Active targeting aims at improving the therapeutic availability of liposomal drugs to target cells within the pathological site and to minimize undesired side effects to non-target cells within the pathological tissue. Although 'active targeting' may suggest that liposomes are actively seeking their targets, resulting in increased amounts of drugs delivered at the diseased sites, this is far from reality. Ligand-mediated binding of liposomes to target cells only occurs when the intravenously administered liposomes 'passively' encounter a target cell. Target cells located in the circulation can be expected to be readily accessible. Target cells outside the vasculature are more difficult to reach and liposomes need to extravasate before being able to bind (80-82). As extravasation of liposome particles is the rate-limiting step in the targeting process, the presence of targeting ligands on the surface of liposomes is not likely to further enhance the amount of drug delivered at the target site. Nevertheless, the presence of targeting ligands on the surface of liposomes can be beneficial by binding to specific target cell receptors leading to improved cellular internalization of the liposomal drug contents. There are different methods available for attaching targeting ligands to PEG-LCL. The most popular methods are based on ligand attachment to the terminal end of PEG (Figure 1) (83-85). For successful ligand-directed targeting of liposomes both the choice of the targeting ligand as well as the cell surface epitope to which the ligand will be directed are critically important.

III.1 Choice of targeting ligand

For active targeting of liposomes any ligand with specificity for cell-surface receptors that are selectively expressed on the surface of the target cell population can be used, as long as chemical conjugation of the ligand to the liposomal surface is feasible without loss of receptor specificity and/or affinity. Frequently used ligands for this purpose are antibodies (86-88), as they can easily be raised against a variety of antigens and often show high selectivity and affinity for their antigen. Besides antibodies other ligands have been studied, such as vitamins (89), peptides (90) and aptamers (91,92).

An important aspect to consider when choosing the appropriate targeting ligand is its immunogenicity. Some ligands, especially those produced in other species, can be recognized as 'foreign' by the immune system of the patient especially when the ligands are conjugated to the distal ends of the PEG chains of LCL (Figure 1) (93). This ligand-mediated immune recognition may oppose the MPS-avoiding characteristics of LCL, resulting in increased clearance rates of i.v.-administered targeted liposomes. For instance, the presence of whole antibodies exposing the constant parts (Fc) of the antibody on the surface of liposomes makes these liposomes highly susceptible to Fc-receptor-mediated phagocytosis by cells of the MPS (94). To prevent this Fc-mediated immune-recognition, antibody fragments such as Fab' and scFv molecules, lacking the constant part of antibody molecules

are frequently used. Important to note is that ligands with low intrinsic immunogenicity may become strongly immunogenic when conjugated to the surface of liposomes. In most cases chemical modification of the targeting ligand is required for covalent conjugation to the liposomal surface. Such modifications may also lead to increased immunogenicity (95).

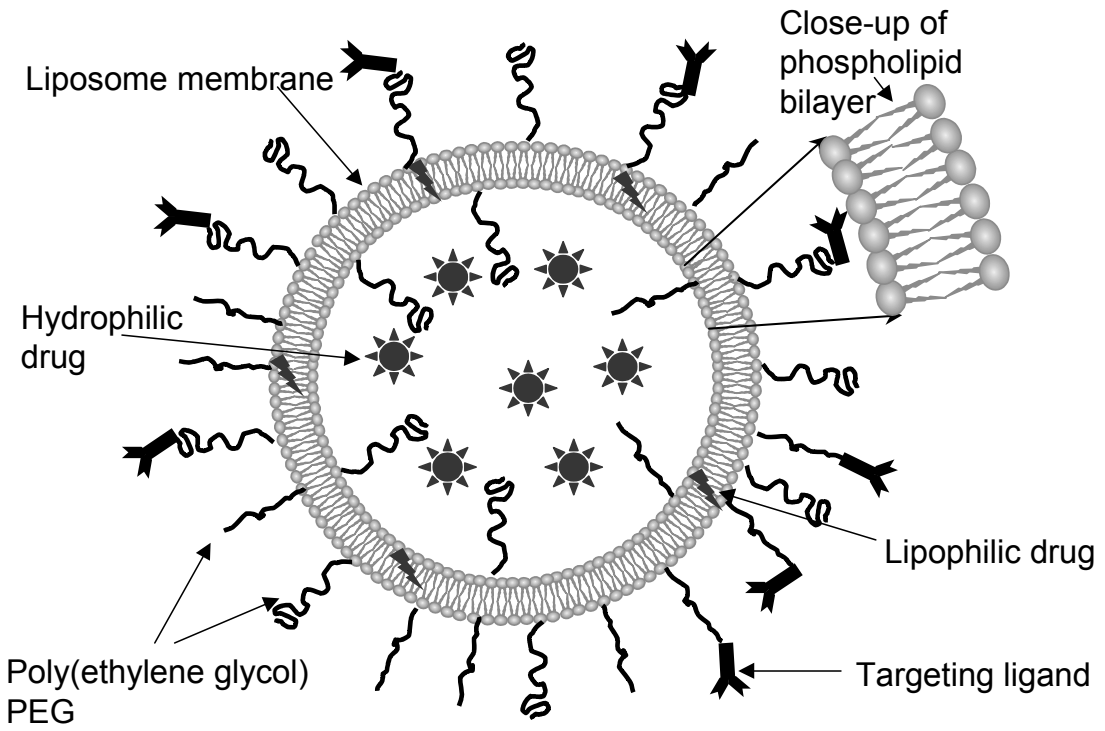

Figure 1. Schematic drawing of a PEGylated liposome with targeting ligands and incorporated drug

Obviously, the affinity of the liposome-conjugated ligand for the target receptor is also an important aspect. Binding to the target receptors should be strong enough to retain the liposomal carrier to the surface of target cells. As multiple targeting ligands are often conjugated to the surface of liposomes, affinity is in most cases not a problem. Even targeting ligands with low affinity for their receptor can be used to obtain strong binding to target cells due to the multivalent character of the targeted liposomes. However, very high affinity interactions between liposomes and target cells should be prevented as this may hamper the distribution of targeted liposomes within the pathological site. This so-called 'binding site barrier' phenomenon has been described for antibodies (96) and scFv molecules (97). Similarly, this phenomenon was offered as an explanation for the observation that immunoliposomes targeted to solid tumors in a nude mice xenograft model were primarily located in the perivascular zones after systemic administration (98,99).

III.2 Choice of target receptor

In choosing the most suitable target receptor, several requirements have to be met. First, the target receptor should be expressed in sufficient amounts to allow accumulation of pharmacologically active drug levels in the pathological tissue. Second, the target receptors should be qualitatively or at least quantitatively different from receptors found in healthy tissue. Although cell surface receptors that are exclusively expressed under pathological conditions are scarce, disease-related overexpression of receptors is often found. For example, overexpression of adhesion molecules is common in inflamed tissue (100,101) and overexpression of growth factor receptors is often found in tumor tissue (102,103). Third, the target receptor should not be shed from the surface of target cells and should be readily accessible to the ligand-directed liposomes. Fourth, if cytosolic delivery of liposomal drug is required, receptor-mediated internalization of liposomes is highly desired (see Figure 2, option 3) It should be realized that targeting of liposomes to receptors with known internalizing capacities does not necessarily guarantee internalization of the liposomes. Binding of the targeting ligand may occur to specific epitopes on the internalizing receptor which do not trigger internalization (104).

III.3 Cytosolic drug delivery

Cytosolic access is problematic with many new biotherapeutic molecules (e.g. proteins, (poly)peptides and nucleic acids). Although ligand-mediated binding of liposomes to cell surface receptors can increase the cellular uptake of liposome-encapsulated drugs, the internalization process itself is not sufficient to yield an enhanced therapeutic effect as long as the entrapped drug is not delivered to the (sub)cellular intervention site. In most cases, the drug needs to be delivered into the cytosol in order to become effective. Some of the delivery strategies leading to cytosolic drug delivery are depicted in Figure 2.

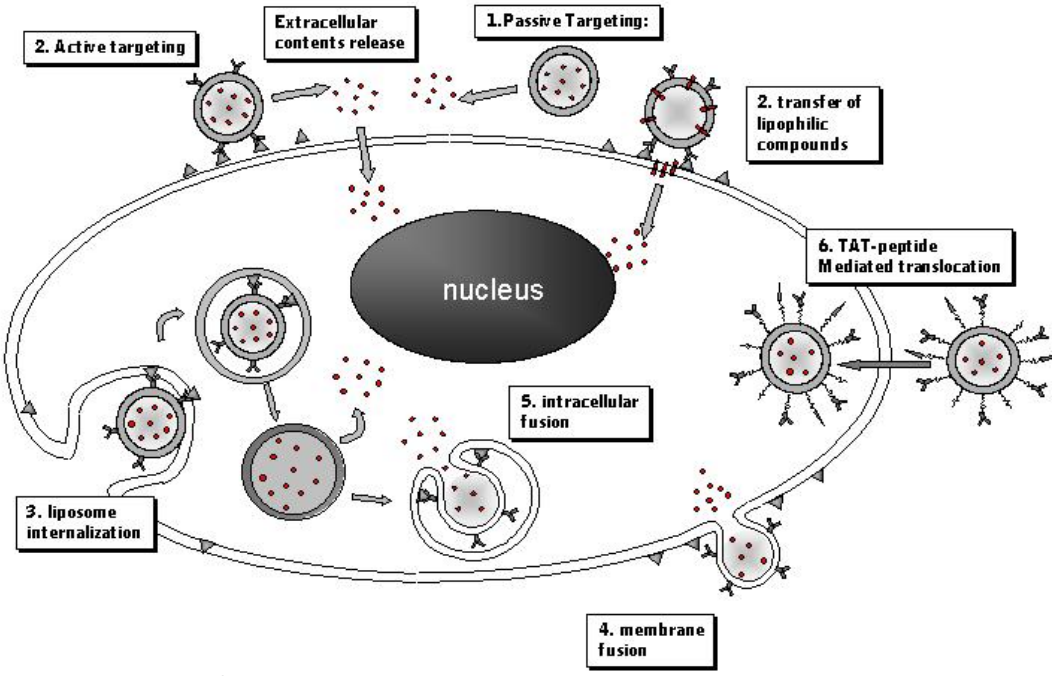

Figure 2. Potential ways of cytosolic drug delivery with passively and actively targeted liposomes

III.3.1 Cell membrane fusion

In case of targeting to receptors that do not internalize the liposomal drug carrier, cytosolic drug delivery can be obtained by fusion of the membranes of the cell-bound liposomes with the plasma membrane of the target cells (Figure 2, option 4). Such fusogenic liposomes have been constructed simply by fusing liposomes with Sendai virus particles. The virus-liposome fusion products retain fusogenic activity and can be used for the cytosolic delivery of liposome entrapped hydrophilic compounds into cells (105,106). Although these fusogenic virosomes have been used for many applications, among which gene delivery and vaccination purposes, specific targeting of these fusogenic vesicles to predefined cell populations remains problematic as the viral receptors present on the virosomes determine which type of cells can be targeted (107,108). Target-sensitive liposomes have been constructed whose bilayers destabilize upon target cell binding (109,110). This binding-induced destabilization results in extracellular release of liposome-entrapped compounds. Therefore, this strategy is not suitable for the delivery of biotherapeutics that require cytosolic delivery but may be useful to improve the therapeutic availability of small, membrane-permeant drugs at target sites.

III.3.2 Endosomal escape

When target cell binding results in internalization of the targeted liposome particles, the majority of the liposomes will face degradation in the endocytic/lysosomal pathway. This delivery route may be useful to obtain cytosolic delivery of drug molecules that can resist lysosomal degradation and diffuse out of the endosomal and/or lysosomal compartments once the liposomes have been degraded as has been reported for doxorubicin (36). However, in many cases delivery into the endosomal pathway results in degradation of the liposome-entrapped drug. To prevent lysosomal degradation and to allow endosomal escape of the liposome-entrapped drug into the cytosol of target cells, endosomolytic functionalities should be incorporated (Figure 2, option 5). These functionalities should induce membrane-perturbing activity preferentially in the low pH environment of endosomal compartments. Several proteins with pH-dependent membrane-perturbing activity have been identified in biological systems and some of them used to obtain enhanced cytosolic delivery are listed below.

Hemolysins. Bacterial hemolysins are proteins with membrane pore-forming capacities that are produced by a variety of bacteria (111). In most cases, the pore-forming activity compromises the integrity of cells resulting in cell death. However, one of these hemolysins, listeriolysin O (LLO) secreted by the intracellular pathogen Listeria monocytogenes exclusively attacks membranes at low pH. Its function is to allow the escape of Listeria monocytogenes from the host's phagocytic vacuoles into the cytosol. Incorporation of LLO into liposomes has resulted in efficient cytosolic delivery of co-entrapped compounds from internalized liposomes without measurably harming the cells (112). As the pores formed by such hemolysins are rather big, ranging in size from 15-35 nm dependent on the type of

hemolysin, this approach will be essentially suitable for the cytosolic delivery of bulky macromolecules such as DNA.

Viral fusion proteins. Several enveloped viruses, among which the human influenza virus A, enter cells by the process of receptor-mediated endocytosis, routing the viral particles into the endosome. The low pH within the endosomes triggers the fusion of viral envelopes with the endosomal membrane, thereby releasing the viral nucleocapsids into the cytoplasm of host cells. In case of the human influenza virus both the adhesion of virus particles to the host cell membrane, which triggers internalization, and the low pH-induced membrane fusion reaction are mediated by the viral spike glycoprotein hemagglutinin (HA) (113,114). The envelopes of influenza viruses have been solubilized, purified and reconstituted into vesicles (115,116). These so-called 'influenza virosomes' bearing both the HA and the neuraminidase (NA) spike proteins retain fusogenic activity exclusively at low pH and have been used as carriers for the delivery of normally membrane-impermeable substances into the cytosol of cells via the endocytic pathway (114-119). However, as these virosomes have a tropism for sialic acid-bearing cells similar to the native virus, targeting of influenza virosomes to specific cell types is hampered. Recently, we have targeted influenza virosomes towards ovarian carcinoma cells by virtue of virosome-conjugated antibodies (Figure 3, unpublished results). This was accomplished by incorporating poly(ethylene glycol) (PEG) conjugated to phospholipids into the virosome membrane. We demonstrated that this PEG-layer on the surface of influenza virosomes shields the interaction of HA with ubiquitous sialic acid residues and at the same time served as spatial anchor for antibody attachment. In this way, virosome binding to cells was exclusively antibody mediated without loss of fusogenic activity. Such antibody-redirected influenza virosomes may be useful carriers for the cytosolic delivery of otherwise membrane-impermeant therapeutic compounds via the route of receptor-mediated endocytosis. Antibody-directed virosomes are expected to be immunogenic due to the presence of viral proteins bearing highly antigenic determinants. This immunogenicity can provide adjuvant-activity when these carriers are used for the delivery of antigens for the purpose of vaccination (120).

pH-dependent viral fusion peptides. The use of pH-dependent fusion peptides represents another approach to obtain cytosolic delivery of biotherapeutic molecules with targeted LCL via the route of receptor-mediated endocytosis (121). Synthetic peptides derived from viral fusion proteins are expected to be less immunogenic than the original fusion proteins as they lack the major antigenic determinants. This is even more true when these peptides are entrapped inside liposomes (122). In addition to reduced immunogenicity, peptides have the advantage that they can be readily synthesized at a large scale without the need for laborious purification procedures. Studies with synthetic peptides resembling the native sequence of the influenza virus N-terminal domain of the HA2 subunit have clearly demonstrated that such peptides are able to destabilize both model membranes (such as liposomes) and natural membranes in a pH-dependent manner (123-125). Fusion peptide-induced lipid

mixing between liposomes has been demonstrated, indicating that these peptides have fusogenic capacities. Influenza virus-derived fusion peptides have been successfully used to enhance the liposomal and endosomal escape of both DNA (122) and bacterial toxin fragments (personal observation) after cellular uptake of targeted liposomal formulations with co-encapsulated fusogenic peptides. This approach of cytosolic drug delivery is particularly effective as it combines targeting with efficient cytosolic delivery. The targeting step provides specificity and ensures delivery of the entire liposomal drug package into the target cells. In addition, the encapsulated fusogenic peptide triggers endosomolytic activity resulting in cytosolic drug delivery.

III.3.3 Membrane translocating peptides

Recently, protein transduction domains (PTD) within proteins have been identified that possess the ability to traverse biological membranes (126). Although the exact mechanism is unknown, transduction occurs in a receptor- and transporter-independent fashion and appears to target the cell membrane directly. Several studies have demonstrated that proteins and even large colloidal particles can be shuttled into cells when conjugated to such PTDs (127). In addition, it was recently demonstrated that conjugation of the protein transduction domain of HIV-TAT to the distal ends of PEG-chains, which were anchored into liposomal bilayers results in translocation of entire liposome particles into target cells (Figure 2, option 6) (128). This transduction process occurred even at low temperatures and in the presence of metabolic inhibitors. Although preliminary, this study shows that PTDs can be used to enhance the intracellular delivery of liposomes into cells. Unfortunately, as the transduction process is independent of specific receptors the PTD-mediated transduction is non-specific and cannot discriminate between cell types. It remains to be investigated whether PTD-mediated transduction of proteins and/or particulate carriers can be limited to specific cell types.

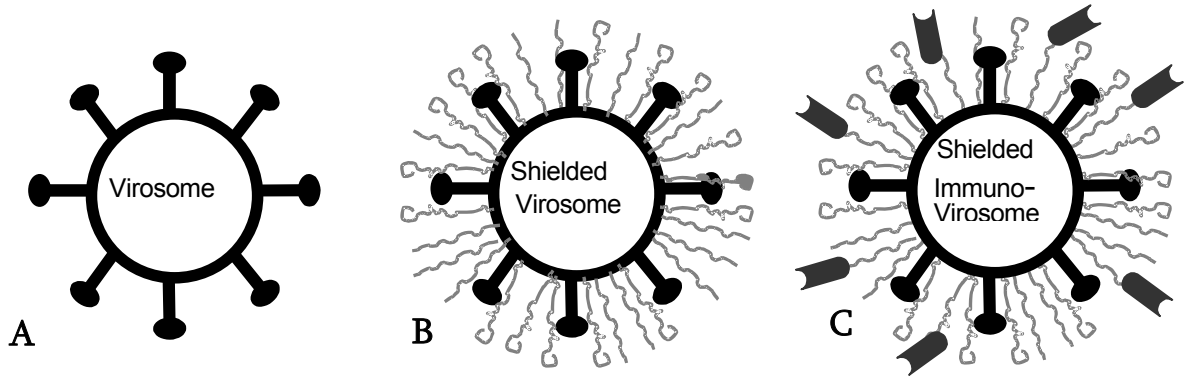

Figure 3. Antibody-redirected targeting of influenza virosomes. Unmodified influenza virosomes expose on their surface many copies of the hemagglutinin membrane protein, which contain the binding pocket for sialic acid residues (A). Poly(ethylene glycol) grafted at high densities on the surface of influenza virosomes can effectively shield the viral spike proteins, thereby preventing HA from interacting with sialic acid residues (B). Conjugation of antibody-Fab' fragments at the distal ends of the surface-exposed PEG chains results in specific binding of virosomes to target cells that is predominantly mediated by the exposed Fab' fragments and not by the HA proteins (C).

IV. FINAL REMARKS

So far, long circulating liposomes appear to offer a range of opportunities for i.v. targeting to pathological sites. The multifaceted capabilities of liposomal formulations seem to continuously provide researchers with new opportunities for drug targeting. The flexibility of the system allows the design and development of liposome systems for the delivery of a wide range of drug molecules: from small stable drugs to larger, fragile biotherapeutics. However, liposomal preparations that are clinically investigated or commercially available mainly exploit the passive targeting effect for reaching tumors or sites of infection/inflammation. Obviously, the more sophisticated targeted liposome designs face a more complicated development route to reach clinical practice. Several complicating factors may play a role: for instance, any additional modification to a given drug carrier system requires thorough investigation of its influence on the safety profile of the delivery system as a whole. Another issue concerns the observation that modification of the surface of long circulating liposomes with targeting ligands or other functionalities often enhances immunogenicity and/or jeopardizes *in vivo* behavior. The latter aspect means that disappointing *in vivo* behavior may obscure each *in vitro* success yielding improved active targeting or cytosolic delivery.

We hope that this review provides the reader with some insight in the design and development of liposomal delivery systems, and envisage that the versatility of the liposomal drug carrier will continue to offer ample opportunities for the development of new and improved liposome systems, applicable in the treatment of life-threatening and chronic diseases.

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CHAPTER 3

JOINT TARGETING OF GLUCOCORTICOIDS WITH LONG-CIRCULATING LIPOSOMES INDUCES COMPLETE REMISSION OF EXPERIMENTAL ARTHRITIS

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ABSTRACT

Objective. To increase the therapeutic activity of glucocorticoids in experimental arthritis by encapsulation in long-circulating PEG-liposomes, which show the ability to preferentially accumulate in inflamed joints after i.v. administration.

Methods. Rats with adjuvant arthritis (AA) were i.v. treated with liposomal and free prednisolone phosphate (PLP) a few days after the first signs of the disease. The effect on paw inflammation scores during the weeks after treatment was evaluated. Liposome biodistribution and joint localization was investigated by labeling the preparation with radioactive ^{111}In. By studying PLP encapsulated in other types of liposomes, which show a distinctive tissue distribution pattern and reduced accumulation in inflamed joints, the importance of targeted delivery to inflamed joints for the increased therapeutic effect was illustrated.

Results. Liposomal PLP proved to be highly effective in a rat adjuvant arthritis model. A single injection of 10 mg/kg resulted in complete remission of the inflammatory response for almost a week. In contrast, the same dose unencapsulated prednisolone did not reduce inflammation, while only a slight effect was observed after repeated daily injections. Evidence was found that preferential glucocorticoid delivery to the inflamed joint is the key factor explaining the observed strong therapeutic benefit obtained with the liposomal preparation, excluding other possible mechanisms like splenic accumulation or prolonged release of prednisolone in the circulation.

Conclusion. Targeted delivery using long-circulating liposomes is a promising, novel means to successfully intervene in arthritis with glucocorticoids.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder, involving joint inflammation and progressive cartilage destruction (1). RA is primarily an inflammatory disease of the connective tissue characterized by spontaneous remissions and exacerbations (flare-ups).

Glucocorticoids can be highly effective in treating joint inflammation, but their systemic application is limited due to a high incidence of serious adverse effects, especially related to long-term treatment (2,3). In addition it is generally assumed that, contrary to the so-called 'disease modifying anti-arthritic drugs', glucocorticoids only suppress the inflammatory process, leaving the progression of disease-related joint destruction unaffected (4,5).

Two aspects are important for the efficacy-safety issues related to systemic glucocorticoid treatment. First is the unfavorable pharmacokinetic behavior of glucocorticoids upon i.v. administration, which is characterized by rapid clearance in combination with a large volume of distribution. Therefore high and frequent dosing is often necessary to achieve an effective concentration of glucocorticoid at inflamed target sites. Combined with the second aspect, namely the profound physiological activity of glucocorticoids in many different tissues, this explains the high risk of occurrence of side effects (6).

We hypothesized that a drug-targeting approach could increase the therapeutic index (7). Glucocorticoids can be incorporated in particulate carriers that show enhanced localization in the target site relative to the drug in unbound form. One of the most interesting carriers for drug-targeting in inflammatory disorders is the long-circulating liposome (LCL) system. Liposomes are small lipid bilayer vesicles (LCL are approximately 100 nm in diameter) with an aqueous core that can be used to entrap water-soluble agents. Often water-soluble polymers like poly(ethylene glycol) (PEG) are attached to the surface of LCL to reduce adhesion of opsonic plasma proteins that would otherwise induce recognition and rapid removal from the circulation by macrophages in liver and spleen (8-10). Using this approach PEG-coated LCL can remain in the circulation with a half-life as long as 50 hours (11,12). Studies with radiolabels entrapped in PEG-liposomes have indicated that PEG-liposomes can selectively extravasate in inflammatory tissues, by virtue of increased permeability of the local vascular endothelium (13,14).

To evaluate whether drug targeting can improve delivery and therefore the efficacy of glucocorticoids, we studied pharmacokinetics, tissue distribution, target localization and therapeutic activity of PLP-PEG-liposomes in a rat model of experimental arthritis. The results indicate that liposomal encapsulation can strongly increase the therapeutic efficacy of PLP. To investigate the critical role of the drug-targeting effect on the improved therapeutic activity, liposome types with short circulation times and reduced localization in the arthritic sites were investigated. The present study shows that enhanced joint accumulation of PLP realized with the long-circulating liposomal formulation explains the increased activity.

METHODS

Preparations

Liposomes were prepared by the film-extrusion method (15). Briefly a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 2.0:1.0. For PEG-liposomes 7.5% of DPPC is replaced by PEG 2000-distearoyl phosphatidylethanolamine (DSPE) conjugate (Avanti Polar Lipids Inc, Alabaster, AL), resulting in a composition containing DPPC, PEG-DSPE and cholesterol in a molar ratio of 1.85:0.15:1.0. A lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml PLP in sterile water. The resulting lipid dispersion was sized by repeated extrusion through a series of polycarbonate filter membranes. Unencapsulated prednisolone was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Small PEG and non-PEG liposomes were sized to a diameter between 90 and 100 nm while the diameter of large PEG liposomes was set between 450 and 500 nm. Phospholipid content was determined with a phosphate assay (16) in the organic phase after extraction liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content. With high performance liquid chromatography using a mobile phase of acetonitril-water with pH of 2 and monitoring the eluens with a UV-detector, which was set at 254 nm, both prednisolone and its phosphate ester could be measured in one single run. The liposomal preparation contained around 5 mg PLP/ml and an average of 60 μmol/ml phospholipid.

Adjuvant Arthritis

The institutional Committee of Animal Experiments approved all animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (200-250 g) were obtained from Maastricht University (Maastricht, the Netherlands). To induce arthritis, 100 ml of incomplete Freund's adjuvant containing 1 mg of heat-inactivated Mycobacterium tuberculosis H37RA (both purchased from DIFCO laboratories, Detroit, MI) was injected intracutaneously at the base of the tail. 10 days after the immunization, the first signs of joint inflammation became apparent, together with a loss of body weight due to the upcoming of the disease. 20 days post-immunization the disease reached maximal severity, after which the inflammation process gradually resolved (18).

Clinical scoring

Starting at day 10, rats were daily weighed and examined for visual signs of inflammation. The severity of the joint inflammation was graded by assigning a score to each paw from 0 to 4, based on erythema, oedema and deformation of the joints. The sum of these four grades for each animal is the clinical score.

Therapeutic efficacy

Rats were treated on day 14 or 15 post-immunization, when the average score of all rats in the experiment reached 7, which is about half the maximal scores reached in these experiments. On the day of treatment, groups of five rats were formed with equal average clinical scores. All preparations were given intravenously via the tail vein. When multiple injections of free glucocorticoid were required, each following day treatment was given at the same time of the day. Clinical score and body weights were monitored daily for up to 2 weeks post-treatment. During the last week of the experiment scoring, was performed less frequently.

Labeling procedure

Liposomes containing diethylene-triamine penta-acetic acid (DTPA) co encapsulated with PLP in the aqueous core were incubated with radioactive ^{111}In-oxine at 60 °C for 1 hr. Efficient transportation of the label through the lipid bilayer at this temperature followed by chelation by DTPA in the liposomal interior resulted in a labeling efficiency exceeding 90% of the total radioactivity added. Non-encapsulated ^{111}In-oxine was removed by gel filtration on a Sephadex PD-10 column. The final preparation contained 120 mCi ^{111}In-label, 40 μmol phospholipid and 3 mg PLP per ml. Since biodistribution studies require less activity than scintigraphic imaging studies, liposomes used for biodistribution were mixed with an equal amount of unlabeled PLP liposomes, in order to keep the total PLP content of each dose constant.

Imaging studies

Whole body scintigraphy was performed with three rats at six time points after injection of 100 mCi ^{111}In-labeled liposomes containing 2.5 mg PLP in the tail vein. Rats were anesthetized and placed prone on a single-head gamma camera equipped with a parallel-hole low energy collimator (Siemens Orbiter, Hofmann Estates, IL). Per image 300,000 counts were acquired and stored in a 256 * 256 matrix.

Biodistribution studies

At four time points (1, 4, 24 and 48 hours) after injection of a dose of liposomes containing 50 mCi ^{111}In-label and 2.5 mg PLP, five rats were euthanized with an overdose pentobarbital. After death, samples of blood, liver, spleen, lungs, kidneys, small intestine, muscles, front and hind paws were collected and counted in a shielded well-type gamma

counter. Injection standards were counted with the tissue samples to correct for physical decay and the percentage of liposome uptake per organ as well as uptake per gram tissue were calculated.

Prednisolone plasma concentration

Blood samples for determination of prednisolone plasma concentrations were centrifuged to obtain plasma and stored at –80 °C. After the radioactivity in the samples had decayed (approximately 30 days), plasma was extracted according to the method reported by Derendorf et al. (17). An HPLC-method similar to that described above was used for the determination of both PLP and prednisolone in the extracts. The detection limit of the method was approximately 20 ng/ml.

Statistical analysis

For comparing clinical scores between groups, the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups with respect to other parameters, one-way analysis of variance was used. P values of less than 0.05 were considered significant.

RESULTS

Pharmacokinetics

Figure 1 A shows that encapsulation of PLP in PEG-liposomes resulted in a change of the pharmacokinetics of the drug. First, the plasma concentration immediately after injection of 5 mg/kg increased from approximately 1 µg/ml to over 100 µg/ml by encapsulating the drug in PEG-liposomes. Calculation of the volume of distribution revealed that PEG-liposomes reduced the distribution volume of PLP from approximately 5 l/kg to less than 50 ml/kg, indicating that PEG-liposomes limit the distribution of the drug to a volume only slightly larger than the plasma volume itself.

Furthermore, liposomal encapsulation strongly reduced the elimination rate of the drug. As unencapsulated ('free') drug in plasma, PLP is rapidly converted into prednisolone. Indeed, neither PLP nor prednisolone were detectable anymore in the plasma samples within 1 hour after injection of the free drug whereas i.v. injection of the liposomal drug yielded an exponential decline of PLP with a half-life of approximately 18 hours. Clearly, the liposomes protected PLP against rapid clearance, and therefore the plasma-concentration time profile of PLP was largely determined by the clearance of the liposome particles. This was confirmed by comparing the radioactivity the plasma samples with the PLP-concentration in the same plasma samples (Figure 1 B). The fact that both PLP and the liposome-associated radiolabel showed the same elimination profile indicated that the majority of PLP measured in plasma remained liposome-associated. Interestingly, despite the stability of the formulation in the circulation, low quantities of free prednisolone could be detected (Figure 1 A).

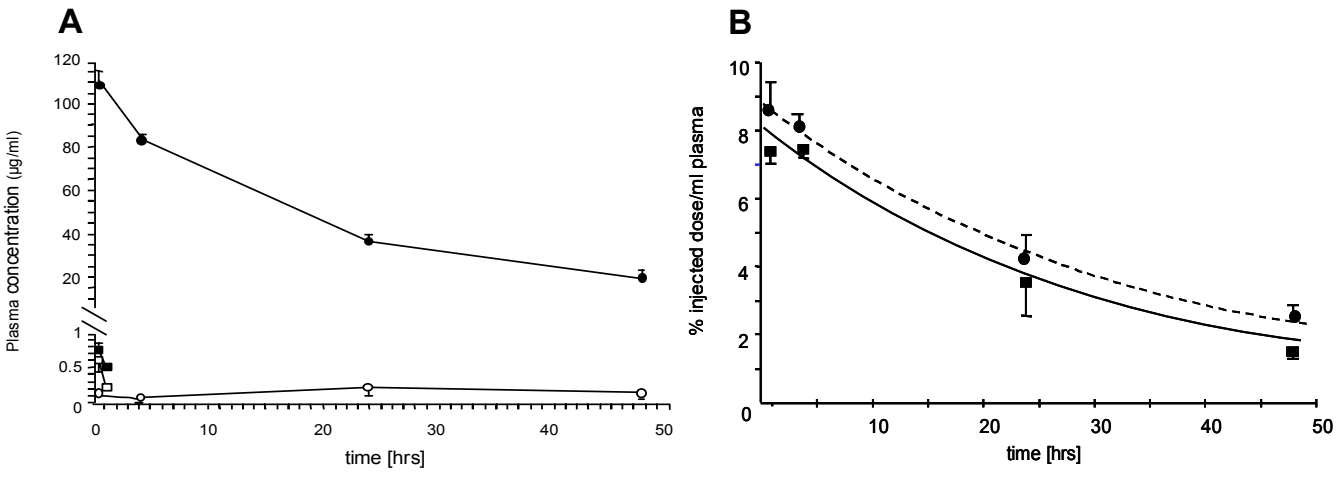


Figure 1. Pharmacokinetics. (A) Plasma concentration of PLP (closed circles) and prednisolone (open circles) after 5 mg/kg liposomal PLP, and PLP (closed squares) and prednisolone (open squares) after 5 mg/kg unencapsulated PLP. (B) % of the injected dose in plasma of liposome label (squares) and encapsulated PLP (circles). Each data point represents the mean of 5 rats ± SD.

Tissue distribution

The scintigraphic images of three rats acquired at different time points after injection of ^{111}In-labeled PLP-PEG-liposomes are shown in Figure 2 A. High blood levels were observed, as reflected by a clearly visible region in the thorax (heart and large vessels), which is in accordance with the results presented in Figure 1. Liver and spleen were also clearly visualized, showing that they represent important organs of liposome uptake. The inflamed joints of front and hind paws became clearly and increasingly visualized at 4, 20, 24 and 48 hrs after injection. This observation points at gradual accumulation and retention of the liposomes at inflamed sites and excludes other possible explanations like increased blood flow in inflamed tissues.

In Figure 2 B quantitative biodistribution data at 48 hrs post-treatment are shown for arthritic and healthy rats. Similar percentages of the injected dose per gram were found in tissues isolated from arthritic rats and healthy rats, with the exception of the paws. Hind paws of arthritic rats had a 7-fold higher liposome uptake than hind paws of healthy rats, indicating localization of liposomes to the inflamed areas.

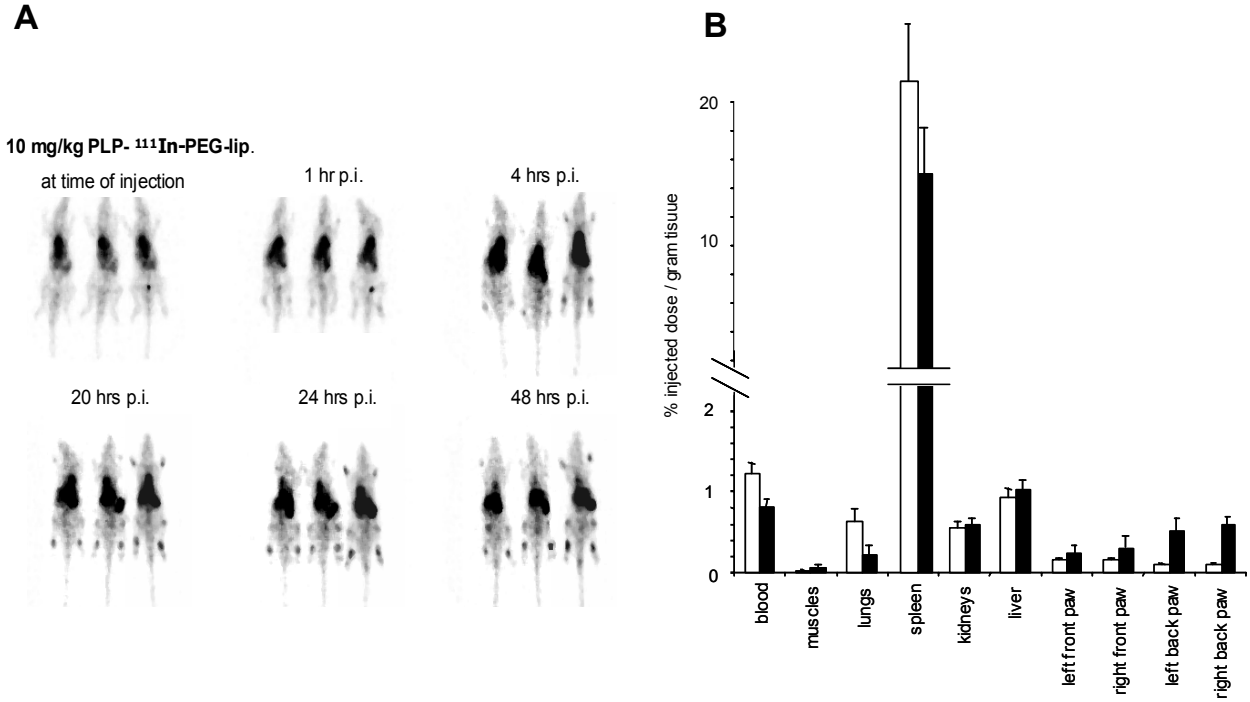


Figure 2. *In vivo* fate of labeled liposomes. (A) Whole body scintigraphic images recorded at 0 to 48 hours post injection of ^{111}In-labeld PLP-PEG-liposomes (B) Organ distribution of ^{111}In-PEG-liposomes in healthy rats (open bars) and arthritic rats (black bars) 48 hours post injection.

Therapeutic activity

Figure 3 A shows the anti-inflammatory activity of free and liposomal PLP at different doses in the rat model of AA. A single dose of 10 mg/kg free PLP did not result in a significant effect on paw inflammation scores. In contrast, the same dose encapsulated in PEG-

liposomes resulted in the complete disappearance of the clinical signs of AA within two days. Resolution of the disease symptoms lasted until day 20 (6 days post-treatment) after which joint inflammation gradually reappeared, reaching the same score as the saline control group around day 28 (two weeks post-treatment). Liposomal PLP was also effective at a 10-fold lower dose (1 mg/kg), and, even at a dose of 0.1 mg/kg, significant suppression of paw inflammation was observed two days after treatment ($p < 0.05$). Only liposomal PLP significantly reversed disease-induced weight loss. Within 8 days these rats completely regained the body weight they lost due to the disease. During the same period, the groups that received saline and free PLP kept loosing weight (Figure 3 B).

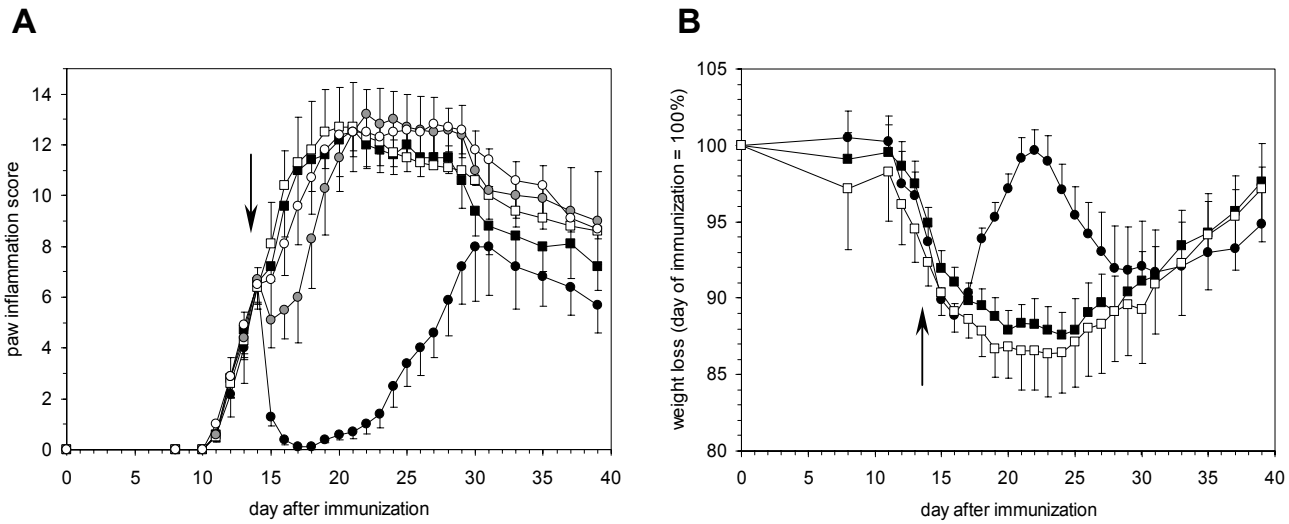


Figure 3. Therapeutic activity in adjuvant arthritis of a single treatment with PLP. (A) Effect on macroscopic inflammation scores of 10 mg/kg (black circles), 1 mg/kg (gray circles), 0.1 mg/kg (open circles) PLP-PEG-liposomes, 10 mg/kg free PLP (black squares), and saline control treatment (open squares) (B) Effect on disease-induced body weight loss of 10 mg/kg PLP-PEG-liposomes (black circles), 10 mg/kg free PLP (black squares), and saline control treatment (open squares). Each point represents the mean of 5 rats ± SEM. Arrow indicates treatment.

Repeated dosing with daily injections of 10 mg/kg free PLP only induced slight reduction of paw inflammation (Figure 4). This dosing regimen was based on clinical experience in RA patients with glucocorticoid 'pulse' therapy (4,5,19) which generally involves 3 to 5 daily injections of 1 gram (methyl)prednisolone. Even though daily treatment of AA rats was continued up to 7 days, no further remission of paw inflammation was accomplished. One day after finishing the last free PLP-injection (day 21), inflammation scores already returned to the average score of the control groups. In contrast, a single injection of liposomal PLP again resulted in almost complete disappearance of joint inflammation until day 20. Figure 4 also shows that control treatment with a single injection of empty liposomes did not have a significant effect.

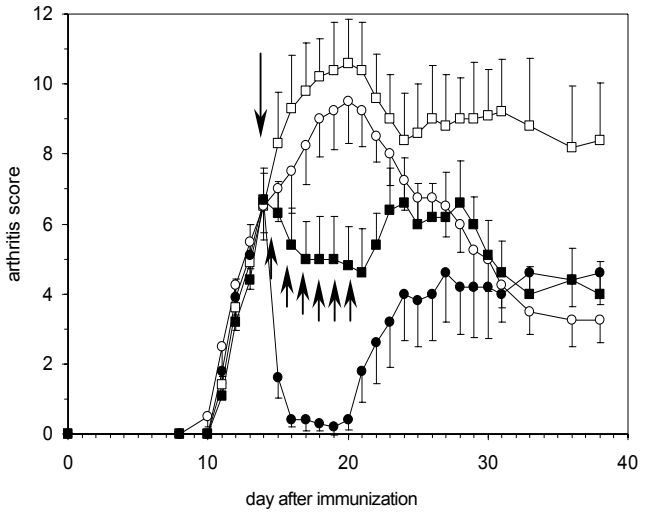
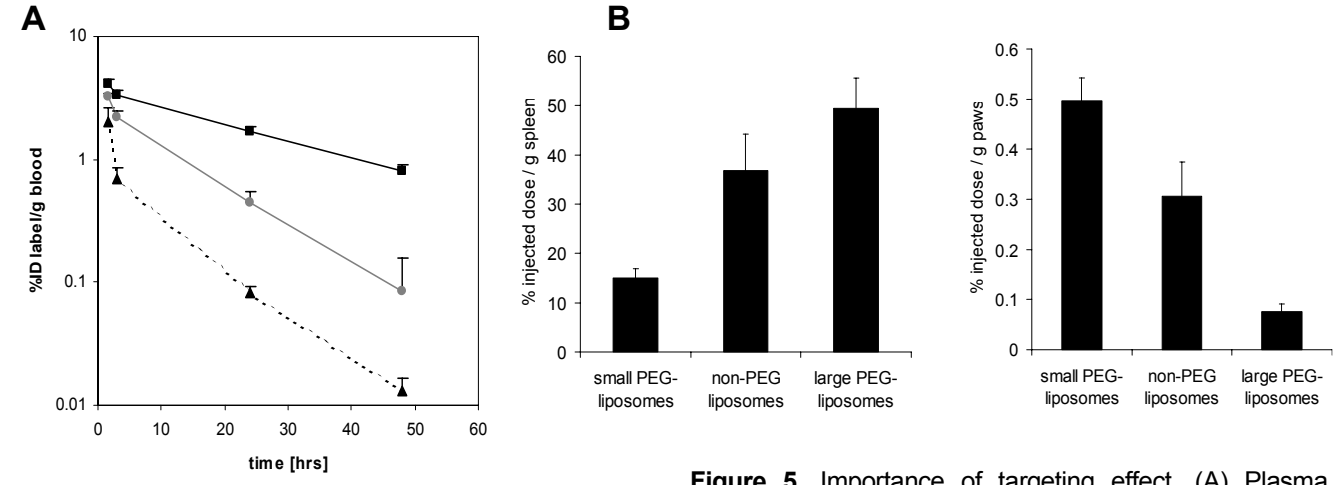


Figure 4. Therapeutic activity of liposomal PLP vs multiple treatment free PLP. Therapeutic activity of a single injection of 10 mg/kg PLP-PEG-liposomes (black circles), and 7 daily injections of 10 mg/kg free PLP (black squares). Control treatments are: empty PEG-liposomes (open circles) and saline (open squares). Each point represents the mean of 5 rats ± SEM. Arrow indicates treatment.

Importance of targeting effect

To study the importance of targeting, two different liposome types with shorter circulation times and reduced joint localization were prepared. (1.) PEG-liposomes with a relatively large mean diameter, referred to as 'large PEG-liposomes', which are known to mainly target to the spleen and (2.) liposomes without the PEG-coating, referred to as 'non-PEG-liposomes', which show enhanced uptake by liver and spleen.



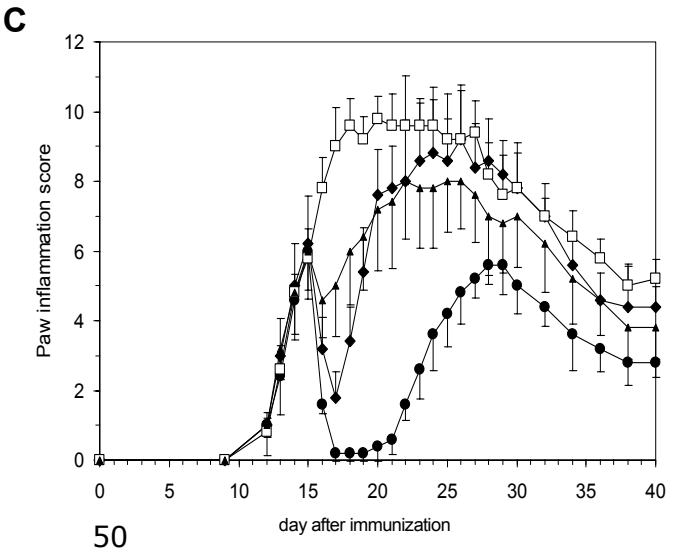


Figure 5. Importance of targeting effect. (A) Plasma concentration-time curves of small PEG-liposomes (black squares), non-PEG-liposomes (gray circles), and large PEG-liposomes (black triangles) Error bars indicate SD. (B) Targeting of the different types of liposomes in spleen (left) and inflamed paws (right). Error bars indicate SD. (C) Therapeutic activity in AA of 10 mg/kg PLP in small PEG-liposomes (black circles), non-PEG-liposomes (black diamonds) and large PEG-liposomes (black triangles) compared to saline as control treatment (open squares). Error bars indicate ± SEM. Arrow indicates treatment. All data in (A), (B) and (C) represent the mean of 5 rats.

In Figure 5 A and B the *in vivo* behavior of both liposome types compared to the small PEG-liposome type is shown. As compared to the small PEG-liposomes, both preparations indeed showed a reduced circulatory half-life with clearly diminished accumulation in the inflamed joints. A much larger fraction of both liposomal formulations was taken up by the spleen.

The therapeutic activity of the three different PLP-liposomal formulations was compared in the AA model. The small PEG-liposomes were clearly the most effective preparation, followed by the non-PEG-liposomes whereas the large PEG-liposomes were only marginally effective (Figure 5 C). Although therapeutically much less active, large PLP-PEG-liposomes completely abolished disease-induced splenomegaly within two days. This indicates that PLP in large PEG-liposomes is potentially equally active as PLP in small PEG-liposomes but that the reduced activity in the joints is caused by reduced localization of PLP in the joints (spleen weight at 48 hrs: 0.38 ± 0.07 g after treatment with large PLP-PEG-liposomes; 0.42 ± 0.04 g after treatment with small PLP-PEG-liposomes versus 1.41 ± 0.19 g in diseased non-treated rats and 0.36 g ± 0.03 g in healthy rats).

Systemic availability

To address the question whether the low quantities of free prednisolone levels observed after injection of liposomal PLP (see Fig 1 A) contribute to the anti-inflammatory effect, prednisolone levels were determined after injection of the three liposome types described above. As shown in Figure 6, the plasma concentration profiles of free prednisolone were not significantly different, despite the clear differences in circulation half-lives and therapeutic activity (see Figure 5). The absence of a clear correlation between the plasma concentration profiles of free prednisolone in the circulation and the anti-inflammatory activity of the three different liposomal preparations does not indicate an essential role of these low systemic glucocorticoid levels in the therapeutic activity of PLP-PEG-liposomes.

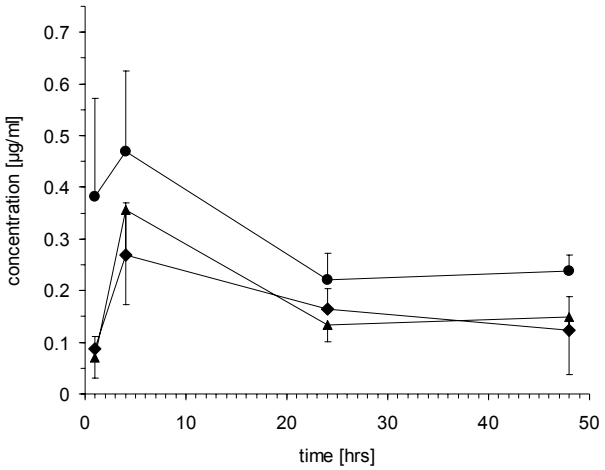


Figure 6. Systemic availability. Plasma concentration-time profiles of free prednisolone after injection 10 mg/kg PLP in small PEG-liposomes (circles), non-PEG-liposomes (diamonds) and large PEG-liposomes (triangles), Each point represents the mean of 5 rats ± SD.

DISCUSSION

The first part of this paper indicates that an i.v. targeted drug delivery approach using long-circulating PEG-liposomes could be a highly attractive alternative to current glucocorticoid treatment strategies, such as i.v. pulse therapy or local intra-articular injections (19-22). Long-circulating liposomes provide the opportunity to achieve high concentrations of glucocorticoid selectively at all arthritic joints by simple i.v. injection. The results indicate that in rat experimental arthritis a single i.v. injection of glucocorticoid-PEG-liposomes can already induce a strong, rapid and long-lasting therapeutic benefit. Complete remission of joint inflammation can be accomplished within two days of treatment, and the therapeutic benefit of the injection lasts for up to two weeks. Furthermore, the results show that an equivalent dose of free PLP is not effective. Limited efficacy, which is certainly not lasting, can be realized only with a treatment regimen involving daily injections of free PLP.

The second part of this paper is devoted to the question of whether the increased therapeutic activity of liposomal PLP can be attributed to drug targeting. The results in this paper indeed point to a crucial role of drug targeting. Encapsulation of PLP in PEG-liposomes strongly influenced the pharmacokinetics of i.v. injected glucocorticoid as shown in Figure 1. I.v. dosing of free PLP in rats resulted in only low and short-lasting plasma concentrations, presumably due to the rapid conversion of PLP into prednisolone. In its turn, free prednisolone was also quickly eliminated, which explains the lack of therapeutic activity of the free drug. Liposomal encapsulation markedly enhanced the concentration of PLP in the circulation. Apparently, liposomal encapsulation protects PLP against conversion and degradation. It also prevents it from the rapid and extensive tissue distribution, which occurs with free PLP. The plasma concentration-time profile of PLP largely resembled the plasma concentration-time curve of a liposome-associated radioactive marker, indicating that leakage of PLP from circulating liposomes does not play a significant role. Since the volume of distribution of PEG-liposomes is much smaller than the free drug, liposomal encapsulation may not just enhance the concentration of drug at the target site, but also lower drug concentrations at non-target tissues. The possibility of reduced toxicity may further improve the therapeutic index of glucocorticoid upon encapsulation in long-circulating PEG-liposomes.

A second confirmation of the crucial role of drug targeting in the enhanced therapeutic activity of liposomal PLP relates to the observation of selective accumulation of the liposomes at arthritic sites (Figure 2). Co-encapsulation of a radioactive marker indicated that the amount of liposomes that is taken up by the inflamed paws increased during the first 24 hrs post-injection, despite decreasing concentrations of liposomes in the circulation. This accumulation process appears to be inflammation-driven, as the degree of joint localization is much lower in healthy rats. Besides the inflamed paws, liver and spleen also appeared to be important organs of liposome uptake in rats with AA. Hepatosplenic uptake is largely responsible for the elimination of liposomes from the circulation and appears to be mediated by resident macrophages in these organs (23,24).

Interestingly, macrophages in lymphoid organs such as lymph nodes and spleen have been shown to be involved in the development of AA (25). The presumed uptake of glucocorticoid-PEG-liposomes by macrophages in the spleen may therefore (partly) explain the therapeutic activity of the preparation. Although it was found that PLP-PEG-liposomes completely reversed disease-induced splenomegaly within two days, the effect on joint inflammation did not relate to spleen targeting, as became evident from the effect obtained with the large PLP-PEG-liposomes. This liposome type, which homed to the spleen to an even higher extent without the capacity to localize in the inflamed joints, completely reversed splenomegaly as well, but only induced a partial remission of joint inflammation. Comparison of the activities of large PLP-PEG-liposomes and small PLP-non-PEG-liposomes to the activity of small PLP-PEG-liposomes, reveals that the therapeutic activity of liposomal glucocorticoid is related to joint accumulation. From our studies it appears that the better liposomes can accumulate in the inflamed joint, the stronger the therapeutic benefit. The process of spleen homing inversely correlated with the process of joint accumulation. Increased spleen uptake may have contributed to the shorter circulation half-life of large PEG-liposomes and liposomes without PEG, resulting in less joint accumulation and consequently in less anti-inflammatory activity. These relationships stress the importance of a prolonged residence time in the circulation for the realization of sufficient joint localization to guarantee strong anti-inflammatory activity of liposomal glucocorticoids.

Besides a strong alteration of pharmacokinetics induced by liposomal encapsulation of PLP, Figure 1 also shows that sustained low levels of free prednisolone in the circulation were generated. Since little to no leakage of PLP from circulating liposomes occurred, it is hypothesized that a certain quantity of encapsulated PLP is released back into the circulation. Possibly, after hepatic and splenic uptake of liposomal PLP, phagocytes in these organs generate and release active prednisolone. The phenomenon of drug release from liposomes mediated by phagocytes in liver and spleen has previously been shown to occur with several liposomal preparations of cytotoxic drugs (26,27). The fact that large PEG-liposomes and non-PEG-liposomes also generated such low levels of free prednisolone supports the hypothesis regarding the drug release by liver and spleen phagocytes. As the therapeutic activity of the latter two liposome types is limited, it is indicated that the phenomenon of sustained levels of active prednisolone in the circulation is not responsible for the increased therapeutic activity achieved by liposomal encapsulation of PLP.

In conclusion, the results in this study indicate that a single i.v. dose of glucocorticoids encapsulated in long-circulating PEG-liposomes can lead to rapid, complete and durable resolution of joint inflammation as a result of enhanced and preferential localization of the liposomal drug in the inflamed joints. This novel approach could offer important advantages over existing therapies in arthritis, such as pulse therapy and intra-articular injections.

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CHAPTER 4

LIPOSOMAL TARGETING OF GLUCOCORTICOIDS TO SYNOVIAL LINING CELLS STRONGLY INCREASES THERAPEUTIC BENEFIT IN COLLAGEN TYPE II ARTHRITIS

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ABSTRACT

Objective. To investigate the effect of a single intravenous treatment with glucocorticoids encapsulated in long-circulating PEG-liposomes on both joint inflammation as well as cartilage destruction and to investigate the phenomenon of selective homing of these liposomes in the inflamed synovium.

Methods. Mice with collagen type II – induced arthritis (CIA) were i.v. treated with liposomal and free prednisolone phosphate (PLP) a few days after the first signs of the disease. Paw inflammation was scored during one week post-treatment, after which sections of the knee joints were prepared for assessment of cartilage damage. In addition, arthritic mice were treated with colloidal gold-containing liposomes. At 24 hours post-injection knee joint sections were prepared in which the location of liposomes was visualized using silver enhancement.

Results. Treatment of CIA with 10 mg/kg liposomal PLP resulted in a complete resolution of paw inflammation. The effect was visible until 1 week post-treatment. 10 mg/kg free PLP could only become slightly effective after repeated daily injections. Although the paw-inflammation recurred at 1 week after treatment with liposomal PLP, knee joint sections prepared at this time point indicated that the cartilage damage was still reduced. Localization of gold-labeled liposomes in the inflamed joints was seen in the proximity of blood vessels, in the cellular infiltrate, but mainly in the synovial lining, which is crucial in the arthritic process. Unaffected joints did not take up liposomes.

Conclusions. This study shows that by using the property of LCL to target the synovial lining selectively in inflamed joints, the anti-inflammatory activity glucocorticoids can be greatly increased, showing also a beneficial effect at the level of cartilage destruction.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder, characterized by joint inflammation and cartilage destruction (1,2). An important role in the pathogenesis of joint inflammation is ascribed to the synovial lining layer that surrounds the connective tissue in the joints. Normally, the synovial lining consists of a few cell layers of mainly fibroblast-like and macrophage-like synoviocytes. In RA however, the synovial lining layer expands as a result of newly arrived macrophages from the periphery (3,4). Macrophages in the synovial lining of arthritic joints have been shown to produce many pro-inflammatory cytokines, attract new inflammatory cells and produce enzymes that can damage the cartilage (3,5,6).

Liposomes were shown to be valuable for targeting macrophages as these cells efficiently phagocytose them upon exposure (7-9). Intra-articular administration of a liposomal form of pro-apoptotic clodronate resulted in selective depletion of the phagocytic lining cells, which resulted in a marked decrease of joint inflammation (10,11). To realize macrophage depletion at inflamed synovia via the systemic route, Camilleri et al. and Richards et al. successfully employed small-sized liposomes that localized in inflamed joints after i.v. injection (12,13,14). However, as the majority of i.v. injected liposomes are generally taken up by the mononuclear phagocyte system (MPS) in liver and spleen, systemic treatment with liposomal clodronate may result in unintended elimination of hepatosplenic phagocytes, which play an important role in the immune defense of the body (15,14).

Effective suppression without elimination of synovial macrophages can be accomplished with glucocorticoids (GC). GC can strongly reduce the generation and release of pro-inflammatory cytokines and cartilage-degrading enzymes by macrophages in arthritic joints (16,17,18). Besides activated macrophages, also the pro-inflammatory activity of fibroblasts, lymphocytes and endothelial cells is suppressed, and this may explain the striking anti-arthritic activity of GC. However, serious adverse effects limit systemic use of glucocorticoids in arthritis patients (19,20). Moreover, high and frequent dosing is necessary to achieve sufficient activity in the joints, since target localization is usually poor as a result of efficient clearance (21).

For efficient delivery of GC into inflamed joints via systemic treatment, the use of small-sized liposomes coated with poly(ethylene glycol) (PEG) may be advantageous. PEG has been shown to be very effective in reducing recognition and rapid removal of liposomes from the circulation by the MPS, enabling liposomes to stay in the circulation for a prolonged period of time (22-24). The long-circulation property provides the liposomes the opportunity to substantially extravasate and accumulate in inflamed tissue (25). A study in arthritis patients treated with ^{99m}Tc-labeled PEG-liposomes indicated that PEG-liposomes can remain in the circulation with a half-life as long as 50 hours and that they selectively extravasate in inflamed joints (26).

The objective of this study was to evaluate the capacity of long-circulating PEG-liposomes to target the inflamed synovium and to expose phagocytic cells in the lining to

encapsulated GC. In the murine model of type II collagen-induced arthritis (CIA) the effect of liposomal GC on paw inflammation was investigated and compared with the effect of unencapsulated ('free') GC. Knee sections were prepared to evaluate the effect of liposomal GC on cartilage damage. Gold-labeled liposomes were used to determine the exact location of uptake in the inflamed tissue and to evaluate whether liposome localization is indeed inflammation-driven.

Our results indicate that selective targeting of the inflamed synovial lining can be realized with long-circulating PEG-liposomes. Encapsulation of GC in these liposomes results in a dramatic increase of the anti-inflammatory activity of GC in the inflamed synovium. Besides a complete resolution of paw inflammation, a significant reduction of cartilage damage still visible at 1 week post-treatment was achieved. The approach of targeted delivery to the inflamed synovium mediated by PEG-liposomes may strongly improve the value of GC in the treatment of arthritis

METHODS

GC-containing PEG-liposomes

Liposomes were prepared by the film-extrusion method (27). Briefly a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), PEG 2000–distearoyl phosphatidylethanolamine (DSPE) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 1.85:0.15:1.0. The lipid solution was transferred to a round-bottom flask and a lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml prednisolone phosphate (PLP) in sterile water. The resulting lipid dispersion was sized by multiple extrusion through polycarbonate filter membranes. Unencapsulated prednisolone was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). The liposomes were sized to a diameter between 90 and 100 nm. Phospholipid content was determined with a phosphate assay (28) in the organic phase after extraction liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content. With high performance liquid chromatography using a mobile phase of acetonitril-water with pH of 2, connected to an UV-detector, which was set at 254 nm, both prednisolone and its phosphate ester could be measured in one single run. Each ml liposomal preparation contained around 4.5 mg PLP and an average of 60 μmol phospholipid.

Gold-labeled PEG-liposomes

Colloidal gold containing PEG-liposomes were prepared as described by Huang et al. with some modifications (29). The method of preparation is similar to the method described above for the preparation of PLP-PEG-liposomes except for the hydration step, which was performed with a freshly prepared tetrachloroaurate solution in citrate buffer. Sizing was performed at 4 °C as described above. Immediately after extrusion, colloidal gold was formed by incubation of the dispersion at 37 °C. To remove non-encapsulated gold, the preparation was eluted on a Sephacryl S1000-SF column (Pharmacia, Uppsala, Sweden).

Collagen-induced arthritis

The Dutch Committee of Animal Experiments approved all animal studies. Male DBA/1lacJ mice between 10 and 12 weeks of age (20-25 g) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). To induce arthritis, 100 mg of bovine type II collagen dispersed in complete Freund's adjuvant containing 2 mg/ml of heat-inactivated Mycobacterium Tuberculosis (both purchased from DIFCO laboratories, Detroit, MI, USA) was injected subcutaneously at the base of the tail. At day 21 a booster injection of 100 mg of bovine type II collagen was given intraperitoneally. At day 24 post-induction, the first signs of joint

inflammation became visible. From that day on, the mice were regularly examined for the visual signs of inflammation. The severity of the joint inflammation was blindly graded by assigning a score to each paw from 0 to 2, based on erythema, swelling and deformation of the joints. The sum of the grades for each animal is the clinical score and varies from 0-8 (30).

Therapeutic efficacy

All mice were treated on day 28 post-induction, when the average score of all mice in the experiment is about half the maximal scores. At day of treatment, groups of 6-7mice were formed with equal average clinical scores. 10 mg/kg PLP both encapsulated in PEG-liposomes and in unencapsulated form was given as a standard dose. This dose was based on clinical experience with so-called 'pulse' treatment of arthritis patients involving single or repeated injections of 1 gram methylprednisolone (31,32). All preparations were given intravenously in the tail vein. Regarding multiple injections of free glucocorticoid, each following day treatment was repeated at the same time during 5 days. The effect of treatment on clinical scores and body weight was monitored 1 week post-treatment.

Histology

At 1 week post-treatment knee joints were dissected and fixed in 4% formaldehyde in phosphate-buffered saline (PBS). In addition the joints were decalcified with 5% formic acid in PBS during 7 days. After dehydration and embedment in paraffin, sections of the knee joint were cut that included patella, femur, menisci and tibia. These sections were mounted on gelatin-coated microscopic slides, stained with hematoxylin and eosin and examined using a light microscope (Leica, DMR, Germany). As a measure of inflammation, infiltrate and exudates were scored by two blind observers. Infiltrate is defined as the influx of leukocytes in the synovium. Exudate is defined as the influx of leukocytes in the joint cavity. The scores varied between 0 and 3, 0 being no cellular influx or exudate whereas 3 indicates maximal cellular influx or exudate. Besides inflammation also cartilage matrix erosion was scored using the same scale, 0 being no cartilage erosion whereas 3 indicates maximal cartilage erosion found at that time-point. Both the tibia and the femur were evaluated.

Visualization of gold-liposomes

A separate group of mice was treated with colloidal gold-containing PEG-liposomes to visualize the exact location of the liposomes in the synovium. 24 hours after treatment knee joints were dissected and decalcified in EDTA/PVP (polyvinylpyrrilodine) in TRIS buffer during 2 weeks. After freezing in liquid nitrogen, sections were cut in a cryostat (Microm, HM500M, Walldorf, Germany). These sections were mounted on Superfrost microscopic slides (Menzel Gläser, Germany). Silver enhancement of colloidal gold was performed with Sigma silver enhancer kit (Sigma, St. Louis, MO, USA) and terminated by incubating with a 0.5% sodium thiosulphate solution in distilled water. The sections were then stained with hematoxylin and eosin and examined using light microscopy.

Statistical analysis

For statistically assessing and comparing therapeutic efficacy in different groups the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups regarding infiltrate and exudate, one-way analysis of variance was used. For statistical evaluation of the effect on cartilage damage Fisher's test was used. P values of less than 0.05 were considered significant.

RESULTS

Therapeutic activity

Figure 1 A shows the anti-inflammatory activity of free and liposomal PLP at a dose of 10 mg/kg in the murine CIA model. A single dose of free PLP did not result in a significant effect on paw inflammation scores. However, the same dose encapsulated in PEG-liposomes resulted in a complete disappearance of paw inflammation at 5 days post-treatment. In Figure 1 B the effect of repeated daily injections of 10 mg/kg free PLP is shown in comparison to 10 mg/kg and 1 mg/kg liposomal PLP. Surprisingly, also repeated administration with 10 mg/kg PLP had only limited effect. The effect is significant compared to the control mice but not significantly different from the effect of a single injection of a ten-fold lower dose of liposomal PLP.

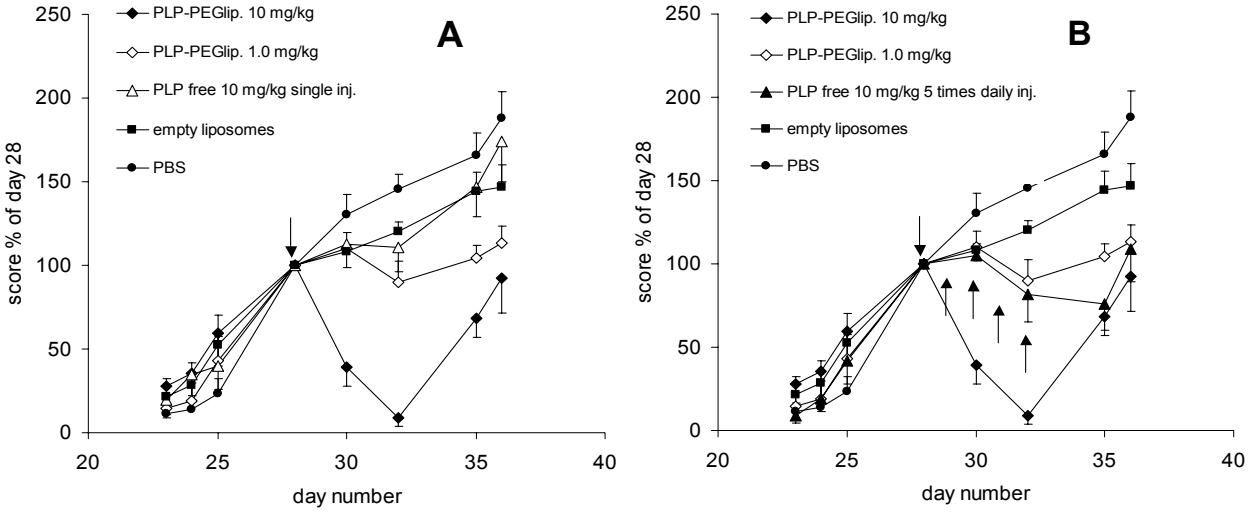

Figure 1. Paw inflammation scores after single treatment with 10 mg/kg PLP-PEG-liposomes (♦),1 mg/kg PLP-PEG-liposomes (◊), 10 mg/kg unencapsulated PLP (Δ), compared to both empty PEG-liposomes (■) and saline (●) as controls. In contrast to liposomal PLP, a single treatment with 10 mg/kg unencapsulated PLP had no significant effect (A). Multiple treatment with 5 daily injections of 10 mg/kg unencapsulated PLP (▲) had significant effect at day 32 and 35 but the effect was not better than 1 mg/kg PLP-PEG-liposomes (B). Each point represents the mean of 7 mice ± SEM. Arrows indicate treatment.

Histological evaluation of inflammation and cartilage damage

Figure 1 shows that 1 week post-treatment paw inflammation scores are recurring. However, the knee sections prepared at this time point still show a profound therapeutic effect of liposomal PLP on the integrity of the cartilage layers (Figure 2). In most of the tissue sections taken from control mice the cartilage appeared highly damaged whereas in the majority of the treated mice loss of cartilage was hardly visible. Figure 3 reveals only little difference regarding infiltrate and exudate as parameters for inflammation. These results correspond with the recurrence of the inflammation score as shown in Figure 1. Scoring of

the cartilage loss, however, revealed a significant beneficial effect in the treated group (Fishers's test, $p < 0.05$). In 4 of the 7 mice treated with liposomal PLP no cartilage degradation was detected whereas in the control group all mice showed profound loss of cartilage.

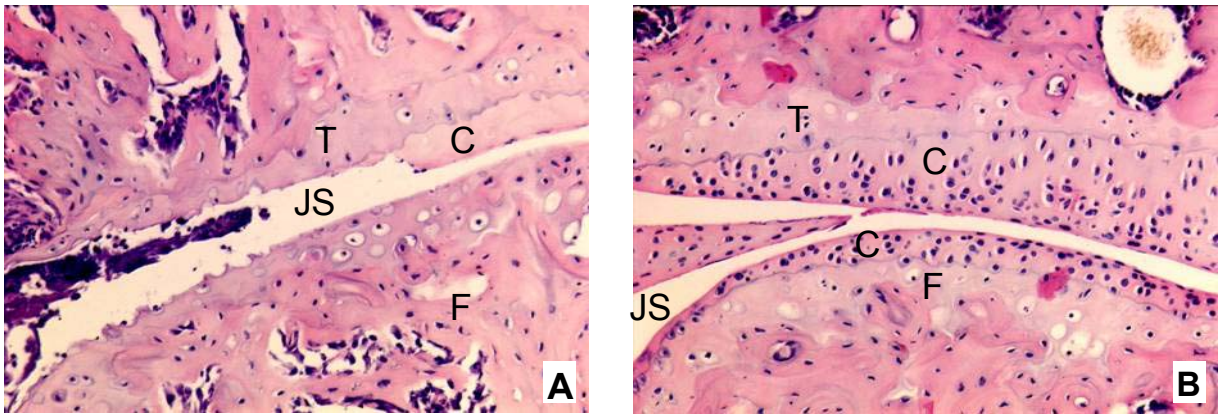

Figure 2. Effect of liposomal PLP on cartilage loss 1 week post-treatment. *(A)* Knee joint section after treatment with saline and *(B)* The same knee joint after treatment with 10 mg/kg PLP-PEG-liposomes. Original magnification x 200. T, tibia; F, femur, JS, joint space; C, cartilage layer.

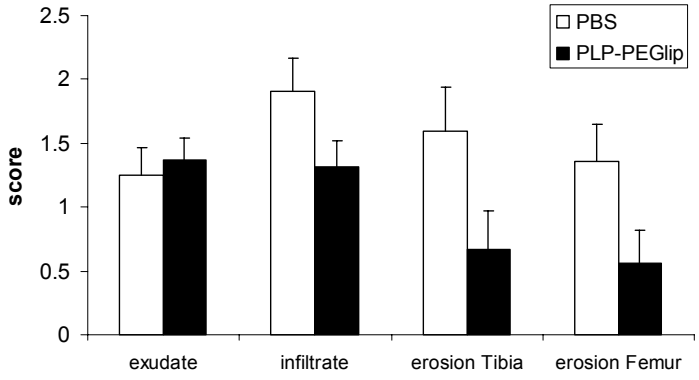

Figure 3. Histological evaluation of the effect on exudate and infiltrate as measures for inflammation and on cartilage erosion of 10 mg/kg liposomal PLP at 1 week post-treatment. Data indicate the mean of 7 mice + SEM.

Localization of PEG-liposomes in the inflamed synovium

The localization of colloidal gold containing PEG-liposomes in the knee sections of CIA-mice was visualized by silver enhancement and shown in Figure 4. Microscopic evaluation revealed that most of the liposomes accumulated in the region of the synovial lining. The majority of visualized gold appeared to be cell-associated. In areas around certain blood vessels the density of liposomes appeared to be relatively high. Despite the severity of the arthritis model, some mice had knee joints without inflammation. Interestingly, hardly any liposomes were visible in joints that did not show signs of inflammation (Figure 5). Clearly, the presence of an inflammatory process is a key factor for the liposomes in order to be able to localize in the synovium.

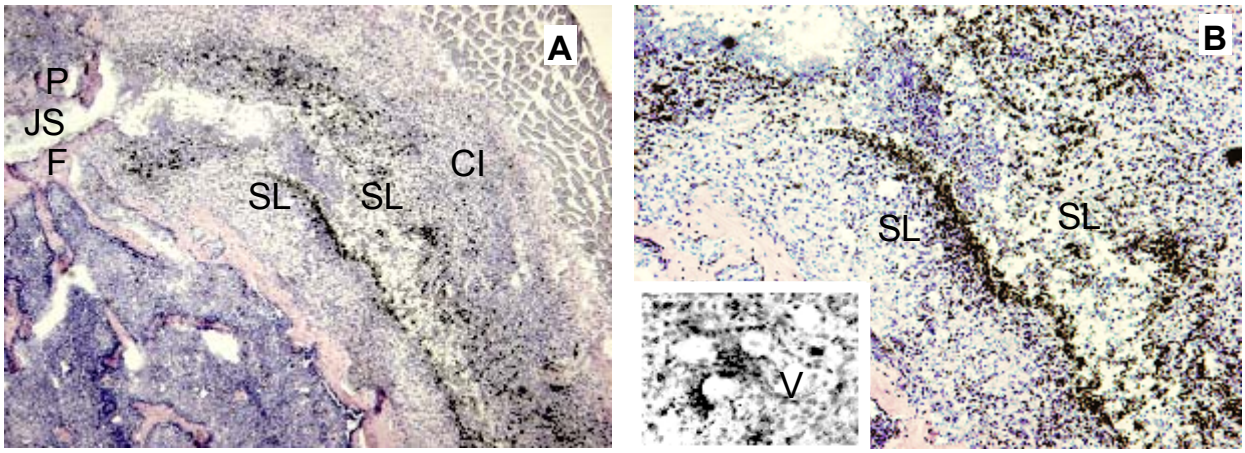

Figure 4. Visualization of gold-labeled PEG-liposomes in the inflamed knee joint. (A) Original magnification X 50. (B) A magnification of X 100 of an area surrounding the synovial lining. Insert: magnification of X 200 of an area surrounding blood vessels. Gold particles are visible as black dots. Note that the liposomal gold is mainly localized in the synovial lining and some around blood vessels. Relatively few gold is visible in the cellular infiltrate. P, patella; F, femur, JS, joint space; CI, cellular infiltrate; SL, synovial lining; V, vessels.

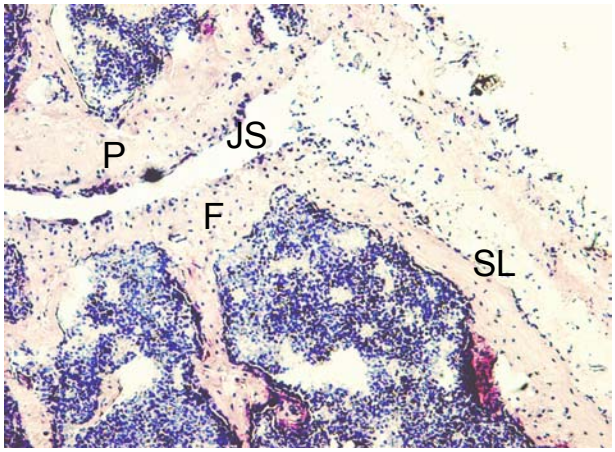

Figure 5. Visualization of gold-labeled PEG-liposomes in an unaffected knee joint. Original magnification X 50. Note the absence of cellular infiltrate and gold-liposomes the synovial lining. P, patella; F, femur, JS, joint space; SL, synovial lining.

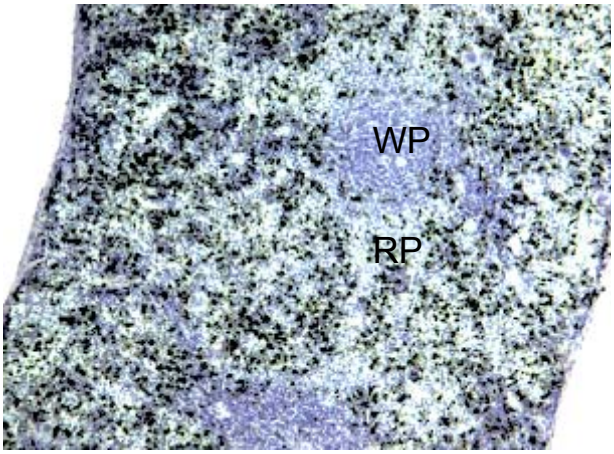

Figure 6. Spleen localization of gold-labeled PEG liposomes. (A) Original magnification X 100. (B) A section of the white pulp surrounded by gold-labeled macrophages that reside in the red pulp. WP, white pulp; RP, red pulp.

Localization of PEG-liposomes in the spleen

Silver enhancement of tissue sections from the spleen shows that almost all liposomes that were taken up by the spleen localized in the marginal zone and the red pulp. Visualized gold appeared to be largely cell-associated. Hardly any gold was detected in the white pulp (Figure 6).

DISCUSSION

In the previous chapter we reported that encapsulation of PLP in long-circulating PEG-liposomes resulted in a strong increase of the anti-inflammatory activity of PLP as compared to the free drug. A single i.v. injection of liposomal PLP could induce a complete resolution of rat adjuvant arthritis. Mechanistic studies with PLP encapsulated in different types of liposomes revealed that the observed increased therapeutic efficacy was a result of the specific property of small long-circulating PEG-liposomes to selectively home to inflamed paws. The present study focuses on the anti-arthritic effect of PLP-PEG-liposomes in murine CIA, which is an experimental arthritis model with a different etiology. This model also allowed a more in-depth investigation of the anti-arthritic effect by histological evaluation of inflamed joints. Furthermore, it became possible to visualize the exact location of the PEG-liposomes inside the inflamed synovium after extravasation from the vasculature.

The data in this study confirm the profound anti-inflammatory activity of PLP-PEG-liposomes. The reduction of paw inflammation scores after a single injection of 10 mg/kg was rapid and complete resolution of arthritis was achieved at day 5 post-treatment. The therapeutic effect lasted for more than a week. 10 mg/kg unencapsulated PLP was only effective after repeated injection but this strategy, which was based on the clinical 'pulse' treatment regimen employed in RA patients, was not more beneficial than a single injection of 1 mg/kg liposomal PLP. Although after 1 week post-treatment joint inflammation clearly recurred, cartilage erosion was still reduced in mice treated with PLP-PEG-liposomes. Apparently, treatment with liposomal PLP results in a profound delay of the cartilage erosion process. This finding may be of importance as treatment of RA with i.v. 'pulse' GC is often criticized for the lack of ability to induce a delay in the progression of joint erosion (33,34).

Knee joint histology performed after injection of gold-labeled PEG-liposomes revealed that the liposomes mainly localized in the synovial lining. Higher magnifications showed that almost all gold particles were cell-associated. The fact that a clear distinction was observed between cells that are positive for gold staining and cells without intracellular gold, strongly suggests that PEG-liposomes selectively localized in cells with phagocytic capacity after extravasation in the inflamed synovium. As it was shown before that gold-labeled PEG-liposomes are stable *in vivo*, it is assumed that all intracellular gold originated from phagocytosed liposomes (29). The affinity of liposomes for phagocytes was also clearly visible in the spleen, in which the phagocyte-rich red pulp showed strong liposome uptake whereas the T cell-rich white pulp showed no sign of liposome uptake at all. As the PEG-coating was used for its capacity to shield liposomes from recognition by MPS phagocytes, the extensive uptake by tissue phagocytes was somewhat unexpected. However, other researchers also report on similar observations with PEG-liposomes targeted to sites of bacterial infection (35,36).

Besides the synovial lining also some gold staining was visible in the infiltrate and around the blood vessels, which brings up two possible explanations for the observed localization of liposomes in the inflamed synovium. First, the gold found in the inflamed

synovium is associated with infiltrating monocytes/macrophages that phagocytosed liposomes in the periphery or second, gold around some blood vessels are liposomes extravasating from the circulation without the help of leukocytes. Schiffelers et al. addressed this issue and found support for the second explanation (35). Also, the fact that Camilleri et al. and Richards et al. report their small clodronate containing liposomes to eliminate synoviocytes despite the depletion of peripheral phagocytes, points in the direction of a phagocyte-independent localization mechanism (12,13,14).

As the onset of CIA varies, some knees of the CIA mice did not yet show signs of inflammation at the day of treatment. Tissue sections from these knee joints did not reveal any sign of liposome localization. Clearly, PEG-liposomes show selectivity for inflamed synovia, which further supports the suggested role of inflammation-enhanced vascular permeability as an essential phenomenon for liposome localization.

The affinity of PEG-liposomes for macrophages in inflamed synovia makes them highly attractive carriers for GC. Macrophages play an important role in the onset and progression of arthritis, as they produce the pro-inflammatory cytokines TNF-α, IL-1, IL-6, generate chemokines, tissue-degrading enzymes and play a role in the presentation of auto-antigens to T cells (5,6). Selective depletion of activated macrophages from the inflamed joint via intra-articular injection of liposomal clodronate was indeed shown to be a promising treatment approach (10,11). Depletion of macrophages via the systemic route appeared to be less attractive, since elimination of macrophages from liver and spleen was shown to strongly affect MPS-functioning (15). GC may be more interesting to encapsulate in liposomes for a systemic treatment approach as GC can effectively downregulate the pro-inflammatory effector production without eliminating the macrophages.

The small PEG-liposomes used in this study offer the advantage of reduced uptake by the MPS, along with a prolonged circulation property resulting in enhanced accumulation at inflamed sites (22-26). The extensive phagocytosis of PEG-liposomes we observed within the inflamed tissue will inevitably result in exposure of the macrophages to high intracellular concentrations of GC, which may be one plausible explanation for the strong anti-inflammatory effect. However, as GC can easily pass cellular membranes, the drug may escape from the intracellular compartment, suppressing other inflammatory cells in the synovium as well. Besides their role as target cell in arthritis, macrophages may therefore also play a crucial role in the release of GC from liposomes and the generation of relatively high and prolonged concentrations of active drug in the synovium. Previously we showed that the liposomal formulation used in our studies was highly stable *in vivo* and that leakage of GC from the liposomes without the involvement of an external trigger is not likely. The suggested need for activated macrophages to release GC from liposomes may add an additional form of target selectivity to the liposomes besides selective accumulation at inflames sites. Further studies are however necessary to address the uptake and intracellular processing of liposomal GC by macrophages.

Our study indicates that the observed property of PEG-liposomes to selectively target the inflamed synovial lining can strongly enhance the beneficial effects of GC in arthritis. The observed affinity of PEG-liposomes for macrophages may lead to high concentrations in inflammatory cells at arthritic joints combined with less exposure of healthy non-target tissues to GC.

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CHAPTER 5

DRUG TARGETING BY LONG-CIRCULATING LIPOSOMAL GLUCOCORTICOIDS INCREASES THERAPEUTIC EFFICACY IN A MODEL OF MULTIPLE SCLEROSIS

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ABSTRACT

High-dose glucocorticoid hormones are a mainstay in the treatment of relapses in multiple sclerosis (MS). We present a way to deliver ultra high doses of glucocorticoids to inflamed sites in the central nervous system (CNS) in experimental autoimmune encephalomyelitis (EAE) using a novel formulation of PEG-coated long-circulating liposomes encapsulating prednisolone (PL). ^{3}H-labeled PL showed selective targeting to the inflamed CNS, where up to 4.5-fold higher radioactivity was achieved than in healthy control animals. HPLC revealed much higher and more persistent levels of prednisolone in spinal cord after PL compared to an equal dose of free prednisolone. Gold-labeled liposomes could be detected in the target tissue, mostly taken up by macrophages (Mφ), microglial cells and astrocytes. Blood-brain-barrier disruption was strongly reduced by 10 mg/kg PL, which was superior to a 5-fold higher dose of free methylprednisolone (MP). PL was also superior to MP in diminishing T cell infiltration by induction of T cell apoptosis in spinal cord. Mφ infiltration was clearly decreased only by PL. The rate of tumor necrosis factor-a (TNF-α) positive T cells or Mφ was strongly reduced by PL and by MP. No adverse effects on glial cells were detected. A single injection of PL clearly ameliorated the course of adoptive transfer EAE and EAE induced by immunization. In conclusion PL is a highly effective drug in treatment of EAE, and is superior to a five fold higher dose of free MP, possibly by means of drug targeting. These findings may have implications for future therapy of autoimmune disorders such as MS.

INTRODUCTION

Multiple sclerosis (MS) is one of the most common inflammatory disorders of the CNS. Its pathological hallmarks are demyelination and cellular infiltration of T cells (TC) and macrophages (Mφ). The most favored pathophysiological hypothesis includes a TC dominated autoimmune reaction (1).

Despite long-term immunotherapy relapses occur, which are commonly treated by repeated i.v. injections of high doses ('pulse') glucocorticoid (GC) as potent anti-inflammatory drug. The main goal is to prevent ongoing tissue destruction with loss of oligodendrocytes, axons and neurons leading to permanent functional deficits. In MS a pulse therapy with 10 mg/kg methylprednisolone (MP) for 3-5 days is the standard regimen in relapse therapy (2, 3). The optimal dosage of the pulse GC is still under debate. Recently, it was reported that treatment with ultra high dose 2 g MP i.v. per day is superior to 500 mg per day for 5 days with regard to reduction of disease activity as measured by MRI criteria (4). In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, we have previously shown, that an ultra high dose of 50 mg/kg MP i.v. is superior to eliminate inflammatory infiltrates than a 'standard' dose of 10 mg/kg MP i.v. and is associated with much higher tissue levels of MP (5).

The pharmacological effects of GC are based on a wide range of mechanisms of action (6). At a lower concentration GC-effects are mainly mediated by the classical GC receptor, yet at a higher concentration additional, non-genomic mechanisms may be operative, such as through membrane receptors and activation of a second messenger system (7, 8). These pathways are thought to be one possible explanation for the observed superiority of high and ultra high doses in the treatment of some autoimmune disorders (6).

The goal of this study was to investigate the effects of long-circulating prednisolone-liposomes (PL), a novel formulation for drug targeting, in treatment of EAE. These PL have been shown to exert a clear beneficial effect in an experimental rat model of arthritis (see Chapter 3). The objective of drug targeting with this formulation is to achieve ultra high tissue concentrations of GC in the inflamed target organ as compared to an equivalent dose given as free drug, and at the same time a much lower concentration systemically with a reduction of unwanted side effects.

Here we show that drug targeting by PL is highly effective in restoring the blood-brain-barrier (BBB)-integrity and in reducing cellular inflammation by induction of TC apoptosis, thereby ameliorating the disease activity of active and adoptive transfer (AT)-EAE without detectable side effects. Moreover, in contrast to free GC, Mφ infiltration was diminished after PL. The effects may be explained by ultra high tissue levels of GC, achieved by means of drug targeting. Our results may have implications for a more efficient therapy of relapses in MS and of other autoimmune disorders.

MATERIALS AND METHODS

Animals, cell culture and EAE

Female Lewis rats (Charles River, Sulzfeld, Germany) were 6-8 weeks old. All culture media and supplements were obtained from Gibco BRL (Eggenstein, Germany). Encephalitogenic TC for *in vivo* experiments were generated and maintained as previously described in detail (9). Briefly, primed TC ($3 * 10^5$/ml) were restimulated with guinea pig myelin basic protein (MBP, 20 µg/ml) in culture medium (RPMI 1640) supplemented with 1% normal rat serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, using freshly isolated and irradiated (3000 rad) thymocytes ($1.5 * 10^7$/ml) as antigen presenting cells.

AT-EAE was induced by tail vein injection of $10\text{-}12 * 10^6$ freshly activated, MBP specific TC. Animals were inspected daily by an observer masked to the respective treatment, using a 6 grade score: 0, healthy; 1, weight loss, limp tip of tail; 2, limp tail, mild paresis; 3, moderate paraparesis, ataxia; 4, tetraparesis; 5, moribund; 6, dead (5). Disease onset in all animals was at day 2, maximum at day 5. Active EAE was induced in rats by immunization with 75 µg guinea pig MBP in 100 µg complete Freund's adjuvant (CFA) per animal by s.c. injection in the hind paws. Disease onset was at day 10 to day 12 with 100% incidence, maximum was at day 13/14.

Preparation of long-circulating PEG-liposomes

Liposomes were prepared by the film-extrusion method (10). Briefly, a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC, from Lipoid GmbH, Ludwigshafen, Germany), poly(ethylene glycol) (PEG) 2000–distearyl phosphatidylethanolamine (DSPE) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 1.85:0.15:1.0. A lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml prednisolone phosphate (Bufa, Uitgeest, The Netherlands) in sterile water. The resulting lipid dispersion was sized by multiple extrusion through polycarbonate filter membranes to a diameter of 90-100 nm. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Phospholipid content was determined with a phosphate assay (11) and prednisolone phosphate concentrations by reversed-phase HPLC (12). Each one ml liposomal preparation contained around 4.5 mg prednisolone phosphate and an average of 60 µmol phospholipid.

Colloidal gold-containing PEG-liposomes (13) were prepared accordingly except for the hydration step, which was performed with a freshly prepared tetrachloroaurate solution in citrate buffer. Immediately after extrusion, colloidal gold was formed by incubation of the liposomal dispersion at 37 °C.

^{3}H-labeled liposomes were prepared similarly except for the lipid composition: to the lipids dissolved in ethanol [^{3}H]-cholesteryloleylether (Amersham, Uppsala, Sweden) was added as a non-degradable liposome lipid phase marker. After rotary evaporation under reduced pressure, the lipid film was hydrated with PBS at an initial total lipid concentration

of 50 µmol/ml. Radioactivity of the liposomal dispersions was assayed in a liquid scintillation cocktail purchased from Ultima Gold (Groningen, The Netherlands) and counted in a Philips PW 4700 liquid scintillation counter. Lipid content of the liposomal dispersion was determined by assessing the loss of radioactivity of the liposomes during preparation. This mixture contained approximately 2.5 mg/ml prednisolone phosphate and 75 kBq/ml radioactivity.

Treatment protocol and tissue sampling

For therapeutic studies we used prednisolone PEG-liposomes (PL) and free prednisolone-phosphate or methylprednisone hemisuccinate (MP) (Urbason solubile®, Aventis, Frankfurt, Germany). Treatment regimen for AT-EAE essentially followed the protocol used in previous studies (5, 14). All experiments, except for gold-labeled liposomes (n=4), and ^{3}H-labeled liposomes (n=4), were performed in groups of 6 animals each and reproduced at least once. 10 mg/kg body weight PL was injected i.v. in a tail vein at 2, 6, 18 and/or 42 hr before perfusion at day 5. As positive control another group received 50 mg/kg body weight MP 18 and 6 hr prior to sacrifice (5).

In a separate experiment colloidal gold-labeled liposomes were applied once at 18 hr or 42 hr. In active EAE in rats 10 mg/kg body weight PL were injected once i.v. at beginning of disease at day 12. 50 mg/kg body weight MP was administered twice i.v. at days 12 and 13. Controls received empty liposomes and/or saline i.v.

For tissue preparation anesthetized animals were perfused with HAES-steril® 6% (Fresenius, Bad Homburg, Germany), followed by paraformaldehyde 4% in 0.1 M phosphate buffer. Spinal cord was removed, postfixed, dehydrated and embedded in paraffin.

For [^{3}H] analysis in EAE vs. healthy rats lethal CO_2-anesthesia was applied and blood was drawn by cardiac puncture and centrifuged. Subsequently, spinal cord, brain, spleen, liver, one sciatic nerve and one 5∗5 mm lower back muscle as non-inflamed control tissue were taken out. All samples were stored at -80 °C until analysis. The organs of one non-treated rat were used for background radioactivity determination.

[^{3}H]-concentrations was measured after homogenization, addition of Solvable tissue solubilizer (NEN, Dreieich, Germany) and 35% hydrogen peroxide. After overnight incubation the samples were assayed in Ultima Gold scintillation cocktail (Packard BioScience B.V., Groningen, The Netherlands). Counting time was to a statistical precision of ± 0.2% or a maximum of 5 min whichever comes first. The scintillation counter was programmed to automatically subtract background and convert counts per min to disintegrations per min. Besides tissue samples also the radioactivity of the injected dose was counted. The ratio tissue samples radioactivity over radioactivity of the injected dose yielded the rate of the injected dose value in percent.

Immunohistochemistry

Five µm cross-sections of spinal cord, spleen or liver were deparaffinized and rehydrated. Pretreatment with hydroxylamine (0.9%, from Sigma-Aldrich Chemicals, Deisenhofen, Germany) was required for albumin stain, and with protease 24 (0.4%, from Sigma-Aldrich) for Kupffer cells. As primary reagents we used: mouse mAb to a pan TC antigen (B 115-1, dilution 1:500, from HyCult biotechnology, via Sanbio, Beutelsbach, Germany) for TC; mouse mAb ED1 (diluted 1:500, from Serotec, via Biozol, Eching, Germany) for Mφ; mouse mAb ED2 (diluted 1:300, from Serotec) for Kupffer cells; rabbit polyclonal Ab to the glial fibrillary acidic protein (GFAP) from cow (diluted 1:500, from DAKO, Hamburg, Germany), incubated at 4 °C over night, for astrocytes; mouse mAb to 2', 3'-cyclic nucleotide phosphohydrolase (CNPase, diluted 1:200, from Chemicon, Hofheim, Germany) for oligodendrocytes; lectin histochemistry with a biotinylated *Griffonia simplicifolia* isolectin B4 (GSA-I-B4, concentration 100 µg/ml, Sigma-Aldrich Chemicals), incubated 24 hr at room temperature, for microglial cells; anti-albumin Ab (diluted 1:200, from Nordic, Bochum, Germany) for detection of BBB-disruption (15). All primary reagents/antibodies were incubated for one hr at room temperature unless stated otherwise. Primary reagents/antibodies were detected using the ABC-system (DAKO), and with 3,3'-diaminobenzidine (DAB) tetrahydrochloride as chromogenic substrate. Sections were counterstained with hematoxylin for 30 sec.

Colloidal gold was visualized with a silver-enhancing solution (Sigma-Aldrich Chemicals) for 16 min in the dark. Sections were fixed by immersing in 2.5% aqueous sodium thiosulfate for 2-3 min, followed by immunohistochemistry for detection of glial or immune cells. Double labeling of apoptotic TC was performed by TUNEL as described before with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as chromogenic substrate (16). Double staining for TNF-α positive TC or Mφ was performed as previously established (13). In brief, TNF-α was detected by a rabbit polyclonal Ab (diluted 1:100, from Serotec), visualized with Vector Red (Vector) as chromogenic substrate, followed by detection of TC or Mφ, using DAB-nickel (black, from Vector) as chromogenic substrate. All sections were dehydrated and mounted in Vitro-clud® (R. Langenbrinck, Emmendingen, Germany). Analysis of inflammatory infiltrates was performed by an observer masked to the respective treatment rating 1.6-3.2 mm^2 of lumbar spinal cord at 250 * magnification. Apoptosis was assessed by morphological criteria (17) or TUNEL. BBB-disruption was quantified by a computer-aided grey scale measuring (Scion Image software, Scioncorp., Maryland, USA) with an Axiovert 100 microscope (Zeiss, Göttingen, Germany) and a CCD DXC 950P camera (Sony, Köln, Germany). We measured the maximal signal intensity of half a spinal cord cross section at 100 * enlargement.

Statistical analysis

Statistical analysis of the data was performed by the Student t test (Excel, Microsoft, Germany), considering *P < 0.05, **P < 0.01, ***P < 0.001 as significant P values.

RESULTS

^{3}H-labeled PL accumulate in CNS.

Selective accumulation of PL in the inflamed CNS was measured by ^{3}H-labeled PL, which were injected at time points indicated in Figure 1 A. The serum level was highest 2 hr after injection and then gradually decreased about 50%, but even 42 hr after injection 3%/g organ of the injected dose was found to be circulating in AT-EAE rats as well as in healthy control animals. In the livers of the rats 1.5%/g organ were achieved after 6 hr, which remained basically unchanged until 42 hr after injection and was similar to levels of a healthy control animal. In spleen 2.7%/g organ were obtained 2 hr after the injection. Furthermore, [^{3}H]-PL accumulated in spleen, reaching 20.3%/g organ 42 hr after injection, which was similar to healthy animals (Figure 1 A). These levels of ^{3}H-labeled liposomes were in accordance with observations in experimental rat arthritis reported by Metselaar et al. (Chapter 3).

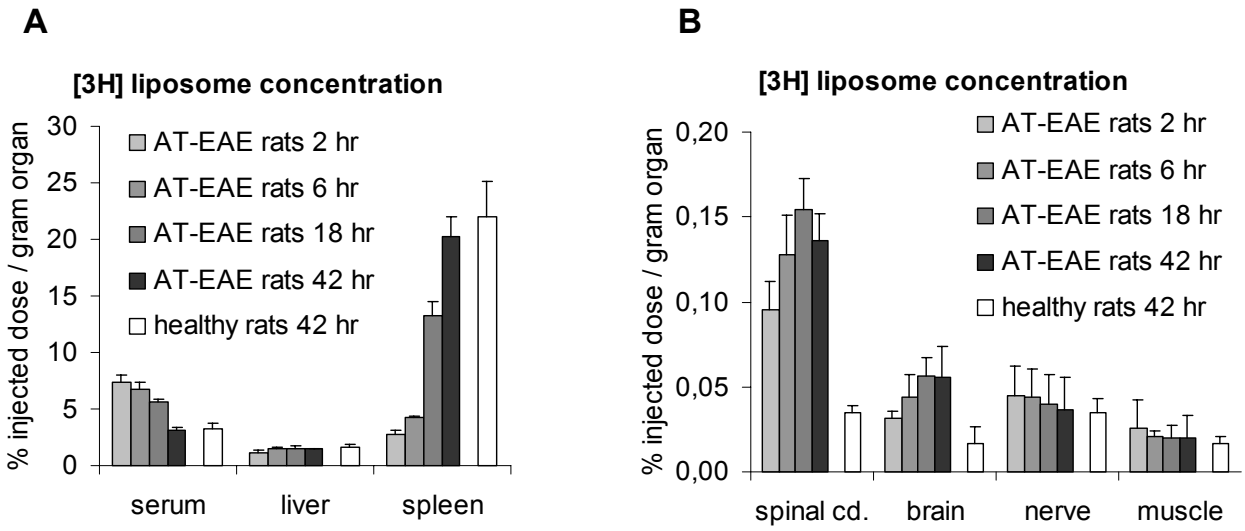

Figure 1. ^{3}H-labeled PL at indicated time points measured in serum, liver and spleen (A) or spinal cord, brain and control tissues nerve and muscle (B). Radioactivity is given as percentage of the injected dose per gram organ of ^{3}H-labeled PL in AT-EAE vs. healthy control rats (n= 4 per group).

The [^{3}H]-values in spinal cord, brain, peripheral nerve and muscle were lower compared to serum, spleen and liver. However, compared to the 42 hr-value in control animals without inflammation, there was a 3-fold higher [^{3}H]-PL accumulation in CNS, in spinal cord with the highest number of inflammatory lesions and BBB-damage (18) even up to 4.5-fold (Figure 1 B). Also, in contrast to nerve and muscle from EAE-rats, only in the inflamed CNS we could observe a gradual increase of the rate of [^{3}H]-PL, indicating an accumulation of PL. In the control nerve and muscle tissue we found no clear difference between EAE-rats and healthy control animals, except for the early time points in the nerve, where a decreasing curve similar to serum was observed. This may be best explained by contamination of the small tissue sample with blood (Figure 1 B).

Single injection of PL is effective in AT-EAE.

First we investigated the optimal timing of the PL injections. We applied a single dose of 10 mg/kg PL i.v. at 6 hr, 18 hr, or 42 hr or empty liposomes at 42 hr before perfusion at day 5 in AT-EAE. 42 hr after PL the rate of TC apoptosis was increased compared to all other groups (***P < 0.001 vs. control or 6 hr; **P < 0.01 vs. 18 hr) (Table 1) and TC infiltration was clearly reduced (**P < 0.01 vs. all groups). PL given at 18 hr before perfusion increased TC apoptosis (**P < 0.01 vs. 6 hr or control), with only slight effect on TC infiltration (Table 1). Only with treatment at 42 hr the disease course was ameliorated, which was repeated once (*P < 0.05 compared to all other groups) (data not shown).

	Apoptosis (%)	Infiltration (TC/mm^2)
Control	12.9 ±4.5	177 ±50
PL 6 hr	15.8 ±2.5	180 ±53
PL 18 hr	22.2 ±3.0**	132 ±24
PL 42 hr	33.2 ±4.2**	42 ± 4**

Table 1. Treatment of AT-EAE with a single i.v. injection of 10 mg/kg PL at indicated time points compared to empty liposomes and perfusion on day 5. Immunohistochemical staining for TC in spinal cord: rate of apoptotic TC (%) rated by morphological criteria and double labeling with TUNEL and number of infiltrating TC (per mm2) (n=5 per group, data are mean ± SD). P-values explained in text.

PL is superior to free GC in AT-EAE.

We then chose the two effective treatments with 10 mg/kg PL at 42 hr and at 18 hr as therapeutic regimen with two injections. This was compared to our previous regimen of two injections of 50 mg/kg MP at 18 hr and 6 hr (5). We investigated the BBB-integrity by an immunohistochemical staining for albumin, followed by a computerized grey-scale analysis. PL (density: 114 ± 1.5) was superior to MP (density: 121 ± 1.9, ***P < 0.001 PL vs. MP); MP also restored the BBB-function as compared to controls (density: 127 ± 2.3, **P < 0.01 MP vs. controls) (2 d-f). PL clearly increased TC apoptosis (***P < 0.001 vs. control, P = 0.07 vs. MP) and reduced TC infiltration (*P < 0.05 vs. all groups) (Figures 3 A,B; 2 a-c). MP augmented the rate of TC apoptosis (**P < 0.01 vs. control), but only marginally reduced TC infiltration. In one experiment we added another group, receiving two injections of 10 mg/kg free prednisolone phosphate. Free prednisolone was less effective than 50 mg/kg MP with regard to TC apoptosis and –infiltration, which is well in accord with previous results (5) (data not shown). Since liposomes are mainly taken up by Mφ as revealed in situ by detection of gold-labeled liposomes, we also characterized Mφ infiltration. The number of infiltrating Mφ in spinal cord was strongly reduced by PL (*P < 0.05 vs. control, ***P < 0.001 vs. MP), whereas MP had no effect. (Figures 3 C; 2 g-i). There was no increase in the rate of apoptotic Mφ, which was in the range of 0 – 5.5% (data not shown). Then we investigated the TNF-α production by TC and Mφ in situ by immunohistochemical double labeling. The rate of TNF-α positive TC in

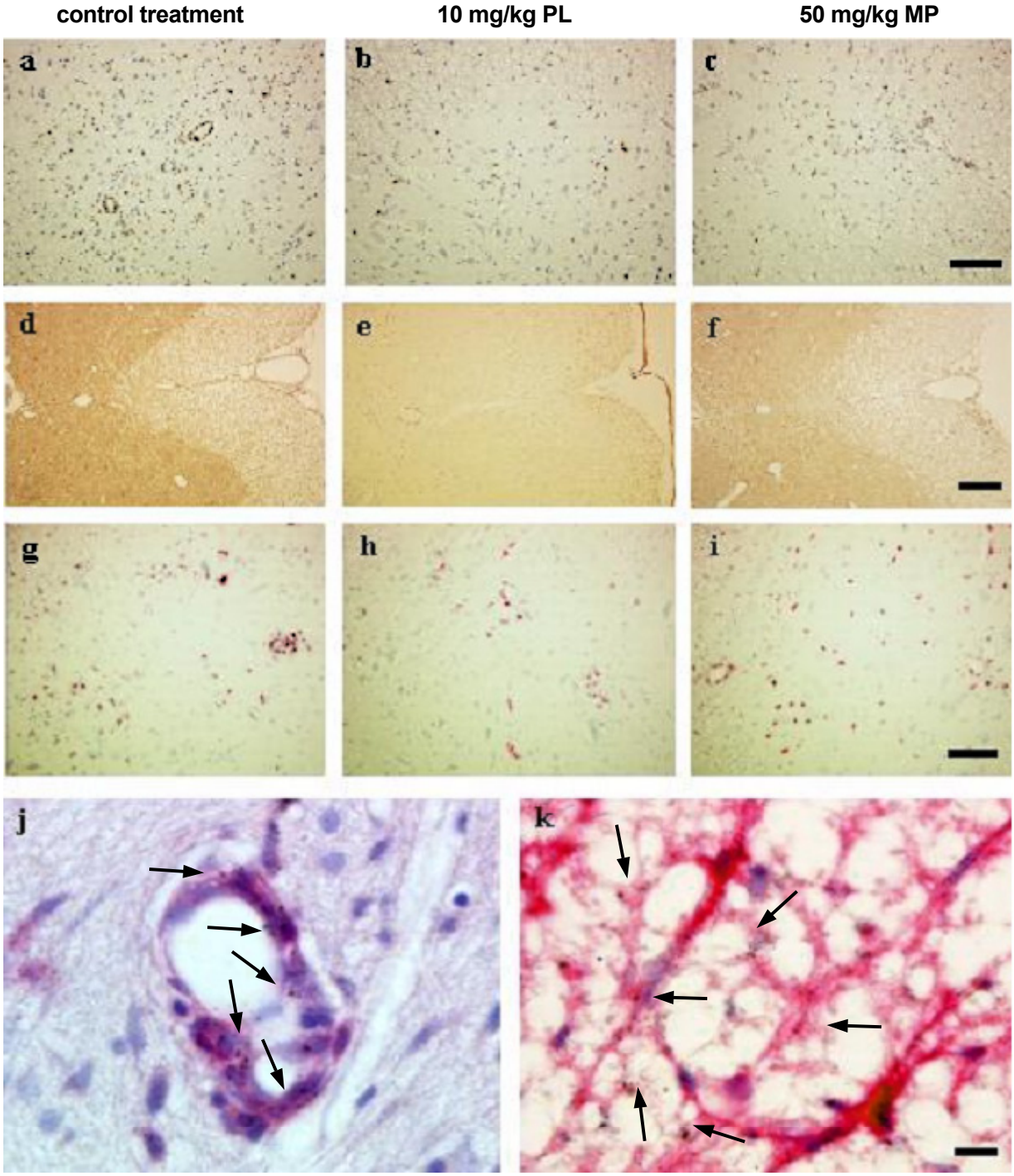

Figure 2. Immunohistochemical detection of TC infiltration, BBB-disruption, Mφ infiltration, and gold-localization in astrocytes and Mφ in 5 µm paraffin sections of spinal cord from AT-EAE rats at day 5. (a-c) Staining of TC (DAB, dark spots) with the mAb B115-1 and hematoxylin counterstaining (gray), in control (a), PL 10 mg/kg (b), or MP 50 mg/kg treatment (c). (d-f) Detection of BBB-disruption with anti-albumin Ab (DAB, dark) and hematoxylin counterstain, in control (d), PL 10 mg/kg (e), or MP 50 mg/kg treatment (f). (g-i) Staining of Mφ (Vector Red, dark spots) with the mAb ED1 and hematoxylin counterstaining (gray) in control (g), PL 10 mg/kg (h), or MP 50 mg/kg treatment (i). (j, k) Detection of gold liposomes by silver-enhancing technique (black dots, indicated by arrows), in combination with staining for Mφ (j, ED1-mAb, Vector Red), or astrocytes (k, anti-GFAP, Vector Red), and hematoxylin. Scale bar=100 µm (a-c, g-i), 200 µm (d-f), 10 µm (j, k).

spinal cord was strongly diminished by PL (32.1 ± 7.8% vs. 55.6 ±8.0% in controls, ***P < 0.001), and was equally effective as MP (34.4 ± 5.9%, ***P < 0.001 vs. controls). Also, the rate of TNF-α positive Mφ was clearly reduced by PL (31.1 ± 7.4% vs. 52.3 ± 4.2 % in controls, ***P < 0.001), and showed the same efficacy as MP (31.1 ± 13.1%, **P < 0.01 vs. controls).

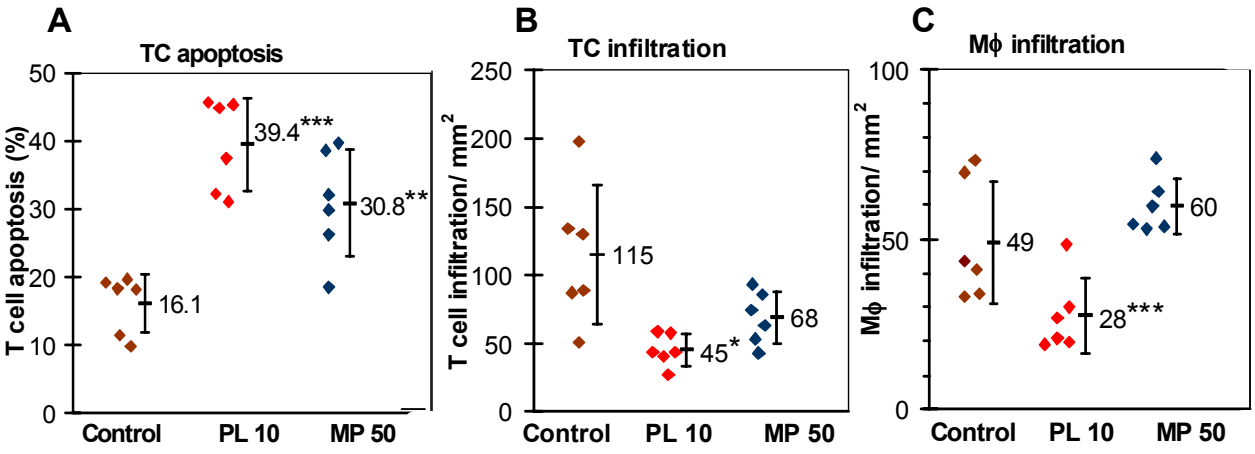

Figure 3. Treatment of AT-EAE with two i.v. injections of 10 mg/kg PL 42 hr and 18 hr, or 50 mg/kg MP 18 hr and 6 hr prior to perfusion on day 5 compared to controls. (A, B) Immunohistochemical staining for TC or Mφ in spinal cord. (A) Percentage of apoptotic TC rated by morphological criteria and double labeling with TUNEL. (B) Number of infiltrating TC per mm^2. (C) Number of infiltrating Mφ per mm2. Each symbol represents one animal (n=6 per group), numbers and bars indicate mean ± SD. P values explained in text.

Even though our study was designed to look for short-term mechanisms in situ, we could also observe a clinical benefit from PL (**P < 0.01 vs. all groups at day 5) (Figure 4 A). However, there was no beneficial effect by MP treatment, which can be explained by the start of treatment at day 4 instead of day 3 in PL. All experiments were reproduced at least once with similar results.

PL ameliorates active EAE.

Since PL appeared to be superior to MP in the different mechanisms investigated and beneficial clinical effects were observed in AT-EAE even with a single injection, we studied if PL could ameliorate the disease activity in an active EAE model. MBP immunized Lewis rats received one injection of i.v. 10 mg/kg PL at beginning of disease on day 12. This was compared to treatment with two injections of i.v. 50 mg/kg MP at days 12 and 13. In this experiment a single PL injection (*P < 0.05 vs. control at day 14) appeared to be equally effective as twice MP at a five fold higher dosage (*P < 0.05 vs. control at day 16) (Figure 4 B).

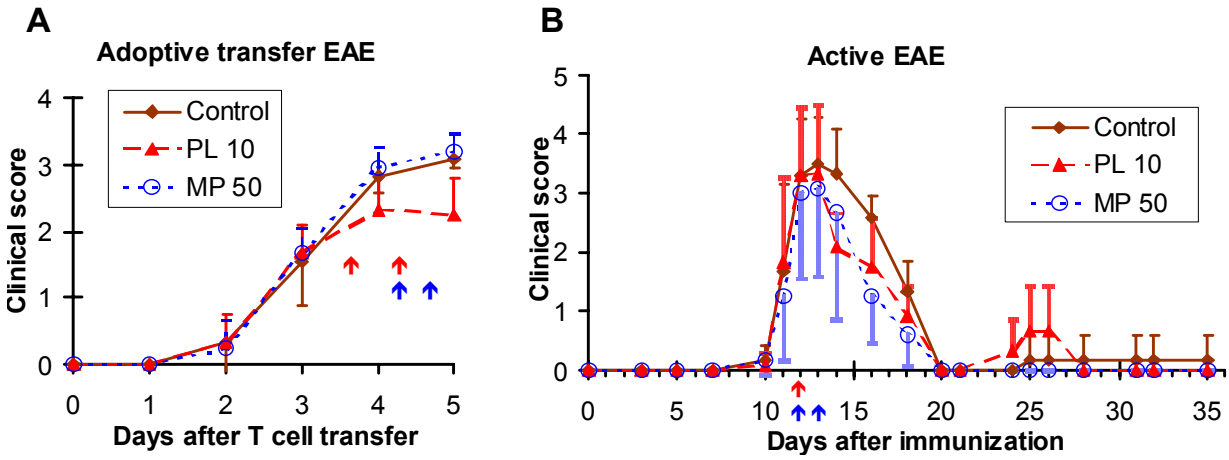

Figure 4. Therapeutic effect on clinical scores (A) Treatment of AT-EAE with a two i.v. injections of 10 mg/kg PL 42 hr and 18 hr, or 50 mg/kg MP 18 hr and 6 hr prior to perfusion on day 5 compared to controls. (B) Treatment of active EAE with one i.v. injection of 10 mg/kg PL at day 12, or two i.v. injections of 50 mg/kg MP at days 12 and 13 compared to controls. Clinical course of disease indicated as mean +/- SD (n=6 per group).

Apoptosis in spleen or liver

We qualitatively investigated apoptosis of phagocytes and TC in spleen and liver by immunohistochemistry. We observed an induction of TC apoptosis in spleen in all PL treated groups, which was stronger as compared to free prednisolone. The frequency of apoptotic TC after injection of free prednisolone compared to controls was elevated after 2 hr and 6 hr, but not after 18 hr and 42 hr. There was no induction of apoptosis or notable change in number of Mφ in spleen and Kupffer cells in liver in any of the treatment groups compared to controls (data not shown).

Gold-labeled liposomes are detected in spinal cord.

To detect the cellular localization of extravasated liposomes, we injected gold-labeled liposomes in AT-EAE rats once 18 hr or 42 hr before perfusion at day 5 compared to unlabeled empty liposomes. As revealed by silver-enhancing technique, gold-labeled liposomes were mostly located within phagocytic cells. Most of the cells were Mφ in the inflamed spinal cord tissue, especially around the blood vessels (Figure 2 j). However, gold label could also be detected in astrocytes and microglia, speaking for a penetration of liposomes through disrupted BBB without the help of Mφ (Figure 2 k). No gold particles were observed in oligodendrocytes. Also, a high amount of gold-labeled liposomes could be detected in Mφ in spleen and in Kupffer cells in liver, which is in accord with other recent findings (Chapter 4). In EAE rats we observed no uptake of gold-liposomes in TC in spinal cord or spleen. There was no silver-enhanced signal in rats treated with unlabeled empty liposomes.

No detectable side effects on glial cells.

Since glial cells can undergo apoptosis, we wanted to examine possible side effects by the high tissue levels achieved by PL. This was especially of note for astrocytes and microglial cells, which appeared to take up liposomes, as revealed by the experiments with gold-labeled liposomes. We characterized astrocytes (GFAP), oligodendrocytes (CNPase), and microglia (GSA-I-B4) immunohistochemically. There was no induction of apoptosis in any of these glial cells and their total number per mm^2 remained basically unchanged after treatment with PL or MP compared to controls (data not shown).

DISCUSSION

Our experiments presented here demonstrate the beneficial effects by drug targeting of prednisolone with a new therapeutic liposomal formulation in the treatment of EAE as a model for MS. Radioactive labeling showed the accumulation of liposomes in the inflamed target organ. By a gold-labeling technique liposomes could clearly be located at the site of inflammation. These results also had functional implications: a dose of i.v. 10 mg/kg PL was superior to a five fold higher dose of i.v. free MP with regard to improvement of BBB-disruption, induction of TC apoptosis, and amelioration of cellular infiltration. Only with PL a clear reduction of inflammatory Mφ in the lesion could be achieved. In addition we observed a reduced rate of TNF-α expressing TC and Mφ *in situ* after PL treatment. As a consequence of the reduced inflammation the disease course of AT-EAE and of active EAE were ameliorated. There were no detectable side effects on glial cells *in situ*.

The therapeutic goal in treatment of MS-relapses is to reduce cellular inflammation as efficient as possible to prevent ongoing tissue destruction and axonal loss. The dosing of GC as mainstay of therapy in MS relapses is still a matter of debate. With regard to our previous findings in EAE (5) one of the major issues of dosing of steroids is to reach very high tissue levels, exerting multiple pathways of steroid actions according to a new model of steroid mechanisms (6). In the present study higher tissue levels of prednisolone were achieved by encapsulation of the steroid in long-circulating liposomes, which delivered the drug to the site of inflammation without a high serum concentration of the free drug.

Conventional liposomes with a relatively short circulation half-life containing hydrocortisone have been developed in the late 70ies for i.a. treatment of arthritis in experimental models and patients (19, 20). In the early 90ies the principle of selective targeting inflamed tissue was reported with cholesterol/lecithin liposomes, which showed an improved stability in the circulation. These liposomes reached the joints after i.v. injection in rats with experimental arthritis (21). Recently it could be demonstrated that a similar formulation could penetrate the BBB in EAE (22). I.v. injected liposomes encapsulating dichloromethylene diphosphonate, which suppresses Mφ activity, were beneficial in EAE and experimental autoimmune neuritis (EAN) (23, 24). For significant accumulation of the encapsulated drug in inflamed extravascular tissue it appears to be crucial that liposomes exhibit a long-circulating behavior, and it seems likely that the effects reported by de Silva et al. and Dingle at al. in the late 70ies were either mediated by a change of the pharmacokinetics of the encapsulated drug or by an indirect effect via monocytes/macrophages in liver, spleen or blood.

Prolonged circulation behavior can be accomplished with small-sized liposomes (< 150 nm) composed of neutral, saturated phospholipids and cholesterol. Often water-soluble polymers like PEG are attached to the surface of long-circulating liposomes to reduce adhesion of opsonic plasma proteins that would otherwise induce recognition and rapid removal from the circulation by the mononuclear phagocyte system in liver and spleen (25-27). Using this approach PEG-coated long-circulating liposomes can remain in the

circulation with a half-life as long as 50 hrs in humans (28). The improved pharmacokinetics and target localization has led to several successful applications of this formulation in antitumor therapy (28, 29). Studies with liposome-associated radiolabels have indicated that PEG-liposomes can also successfully be employed to selectively target pathological sites in inflammatory disorders (30, 31). For our study here we encapsulated prednisolone-phosphate as active drug into long-circulating liposomes, since methylprednisolone-succinate, which we used in previous studies (5, 13), did not yield a stable formulation when encapsulated in PEG-liposomes.

To investigate whether target localization of liposomes is a direct process or a result of uptake by monocytes in blood or spleen followed by infiltration of such monocytes at the target site, we employed gold-labeled liposomes, which could be detected in spinal cord within vascular endothelium and perivascular areas non-phagocytosed as well as in inflammatory Mφ. Additionally, there was direct uptake of liposomes by resident astrocytes and microglia. Also, our radioactive data support a rapid penetration of liposomes into the CNS, where an accumulation of the injected liposomes was seen, reaching values of up to 4.5-fold higher than in healthy control animals. In contrast, non-inflamed control tissue showed similar amounts of radioactive liposomes in healthy animals and EAE-rats. Taken together these data support the hypothesis of selective targeting of PL to inflamed sites.

The theoretical prednisolone concentrations calculated based on concentrations of radioactive PL found in the spinal chord are in the range of non-genomic effects according to a new model of steroid mechanisms of action (6). These expected high levels of prednisolone after PL may have been responsible for the direct effects at the site of inflammation, such as the superior induction of TC apoptosis and the superior improvement of the BBB function. The TNF-α expression of TC and Mφ was reduced comparably to a 5-fold higher dose of free MP. However, besides clear direct effects, the reduced infiltration of TC and Mφ in the spinal cord may additionally be due to effects of PL in peripheral immune organs. We could not detect augmentation of apoptosis of Mφ in spleen. Despite high organ concentrations of PL we could not detect apoptosis in resident cells like Kupffer cells in liver or astrocytes, oligodendrocytes, or microglia in spinal cord, which rules out some important unwanted side effects. One could even speculate that the immune cells playing an active role in the disease are more susceptible to a high-dose steroid treatment than resident or resting immune cells. Also recent observations in exp. arthritis did not reveal unwanted side effects of PL.

Taken together we show that long-circulating PL given at 10 mg/kg accumulate in the inflamed organ of EAE rats. Augmentation of TC apoptosis *in situ* occurs rapidly and the BBB function is improved. The reduced infiltration of TC and Mφ ultimately leads to an ameliorated disease activity of active and AT-EAE. Especially the reduced Mφ infiltration, which was only seen after PL, might help to prevent ongoing tissue destruction. Thus, PL could be a therapeutic alternative to free MP, which even at a 5-fold higher dose remains less effective. Finally, employing the principle of drug targeting may reduce systemic side effects. These findings may have implications for the treatment of inflammatory disorder of the CNS such as multiple sclerosis and other autoimmune diseases.

ACKNOWLEDGEMENTS

The authors thank Prof. Rudolf Martini for stimulating discussions. Gabriele Köllner, Helga Brünner and Verena Wörtmann performed excellent technical assistance. Louis van Bloois is gratefully acknowledged for his help with preparing the liposomes. J.S. is a recipient of a grant from the Deutsche Forschungsgemeinschaft (DFG-grant Schm 1669/1-1).

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CHAPTER 6

LIPOSOME-ENCAPSULATED PREDNISOLONE PHOSPHATE INHIBITS TUMOR GROWTH IN MICE

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ABSTRACT

Glucocorticoids can inhibit solid tumor growth, which has been suggested to be caused by an inhibitory effect on angiogenesis. Several mechanisms have been proposed to explain their anti-angiogenic action, ranging from inhibition of endothelial cell proliferation and migration, modulation of basement membrane turnover, and/or inhibition of production of pro-angiogenic factors. The anti-tumor effects of the free drugs have only been observed using treatments schedules based on high and frequent dosing for prolonged periods of time. As long-circulating liposomes (LCL) accumulate at sites of malignancy, we investigated the tumor-inhibiting potential of LCL-encapsulated prednisolone phosphate. It appeared that liposomal prednisolone phosphate could inhibit tumor growth dose-dependently. 80-90% tumor growth inhibition was achieved in s.c. B16 melanoma and C26 colon carcinoma murine tumor models at a dose of 20 mg/kg by single or weekly doses. Prednisolone phosphate in the free form was completely ineffective at this low frequency treatment schedule, even at a dose of 50 mg/kg. Histological evaluation revealed that liposomal PLP-treated tumors were surrounded by a layer of connective tissue, whereas the tumor center contained areas of apoptotic cells. In addition, blood clots were observed in some of the larger blood vessels in these tumors. In conclusion, the present study shows the potent anti-tumor efficacy of a new, liposomal, formulation of glucocorticoids.

INTRODUCTION

Glucocorticoids (GC) have a wide spectrum of activities on cell trafficking, cell-cell interactions and cell communications, leading to pronounced anti-inflammatory and immunosuppressive effects. GC exert their effects by diffusion through the cell membrane and binding to their cytosolic receptors. Subsequently, these receptors become activated and translocate to the nucleus where they directly modulate DNA transcription of a variety of genes. In addition, GC-receptors may directly or indirectly antagonize the activity of several transcription factors, most notably nuclear factor kappa-B (1,2). GC also exert rapid non-genomic effects on cells by interacting non-specifically with cellular membranes, or specifically with membrane bound GC-receptors (3).

In tumor therapy, GC have been used for their anti-inflammatory and anti-emetic effects and for the treatment of hematological malignancies based on their efficient cytolytic activity on cells of lymphoid origin (4). Reports in the last two decades demonstrated that GC could also decelerate solid tumor growth in experimental animal models (5-8). However, one of the drawbacks of GC in tumor therapy, as shown in these pre-clinical studies, is the need for high or frequent dosing. In mice, doses of 100-200 mg/kg per day need to be administered for prolonged periods of time to obtain significant tumor growth inhibition (5-8). Moreover, these doses have been shown to cause considerable morbidity and mortality as a result of severe immune suppression in experimental animals (6,7).

Targeted delivery of GC to tumor tissue could be an attractive strategy to increase intratumoral drug concentrations, thereby reducing the overall dose decreasing the likelihood of side effects (9). In the present study, we investigated the use of long-circulating liposomes (LCL) to deliver GC selectively to tumor tissue. LCL have previously been shown to accumulate at sites of malignancy as a result of the enhanced permeability of tumor vasculature as compared to healthy endothelium (10). Furthermore, in previous studies in our group, the therapeutic activity of encapsulated in LCL was shown to be strongly increased in experimental models of arthritis (Chapter 3 and 4). In the present study, anti-tumor activity of liposomal prednisolone phosphate (PLP) was investigated in s.c. C26 colon carcinoma and B16F10 melanoma models and compared to the anti-tumor activity of free PLP in different dosing schemes.

MATERIALS AND METHODS

Liposome preparation

LCL were prepared as described previously (11). In brief, appropriate amounts of dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly(ethylene glycol) 2000-distearoyl phosphatidylethanolamine (PEG-DSPE) (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in chloroform:methanol (2:1 vol:vol) in a round-bottom flask. A lipid film was made under reduced pressure on a rotary evaporator and dried under a stream of nitrogen. Liposomes were formed by addition of an aqueous solution of 100 mg/ml PLP. A water-soluble phosphate ester of prednisolone was used to ensure stable encapsulation in the liposomes. For labeling of the liposomes with 0.5 mCi ^{111}In-oxine (Mallinckrodt Medical, Petten, The Netherlands), the lipid film was hydrated in 5 mM diethylene-triamine penta-acetic acid (DTPA) in 10 mM N-(-2 hydroxyethyl) piperazine-N'-ethane sulfonic acid (HEPES)/135 mM NaCl-buffer pH 7 to a final lipid concentration of 10 µmol/ml, according to a procedure described by Boerman et al. (12). The liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm.

Short-circulating liposomes (SCL) were prepared similarly, only PEG-DSPE was replaced by egg phosphatidylglycerol (EPG). SCL were used to determine the dependency of anti-tumor effects on the degree of liposome localization in the tumor, which is positively correlated to liposomal circulation time (10). Therefore, PEG-DSPE was exchanged, as it is responsible for the prolonged circulation of the LCL by providing a layer of steric stabilization around the liposome surface, decelerating MPS uptake. Moreover, exchanging it for EPG introduced a negative charge on the liposome surface, which promotes MPS-uptake and thereby reduces circulation time of the SCL even further (13). The SCL were formed by addition of 10 mg/ml PLP in 10 mM HEPES/135 mM NaCl-buffer pH 7.4 to the lipid film, and extrusion took place through polycarbonate membranes of 400 nm. Unencapsulated material was removed by dialysis with repeated changes of buffer against 10 mM HEPES/135 mM NaCl-buffer pH 7.4 at 4 °C.

Mean particle size of the LCL was determined by dynamic light scattering and found to be 0.1 µm with a polydispersity value of around 0.1, whereas the SCL had a mean particle size of 0.5 µm with a polydispersity value of around 0.3. The low polydispersity values indicate limited variation in particle size. The large size of the SCL also reduces circulation time as compared to the LCL and also prevents efficient extravasation at the target site (13,14). Taken together, the changes in lipid composition and liposome size strongly reduce the degree of liposome localization in the tumor for SCL as compared to LCL.

Phospholipid content was determined with a phosphate assay, performed according to Rouser (15), on the organic phase after extraction of liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content by high performance liquid chromatography using a mobile phase of acetonitril-water with

pH of 2 and monitoring the eluens with a UV-detector, which was set at 254 nm. The liposomal preparation contained around 2 mg PLP/ml and 60 μmol/ml phospholipid. Using this setup it was established that liposomes contained 25-35 μg PLP/μmol lipid.

Cells

B16 murine melanoma and C26 murine colon carcinoma cells were cultured at 37 °C in a 5% CO_2-containing humidified atmosphere in culture medium (DMEM) (Gibco, Breda, The Netherlands) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Gibco). To determine whether prednisolone (phosphate) had a direct cytotoxic effect on tumor cells, 10^4 cells/well were plated in a 96-well plate. Prednisolone (PL) was added dissolved in ethanol, using corresponding concentrations of ethanol as controls, whereas PLP was added in HEPES/NaCl-buffer. Cell viability was determined after 24 h and 48 h of incubation by XTT-assay (Sigma, St. Louis, USA) according to manufacturer's instructions.

Murine tumor models

Male Balb/c and C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (the Netherlands) kept in standard housing with standard rodent chow and water available *ad libitum*, and a 12 h light/dark cycle. Experiments were performed according to national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16 melanoma or C26 colon carcinoma cells were inoculated subcutaneously in the flank of syngeneic C57Bl/6 or Balb/c mice, respectively.

Tissue distribution of ^{111}In-labeled LCL in tumor bearing mice

At a tumor volume of approximately 1 cm^3, mice were injected i.v. with 25 μmol lipid/kg (corresponding to 30 x 10^6 cpm/mouse) of ^{111}In-labeled LCL. At 6 h and 24 h after injection animals were sacrificed, a blood sample was taken and tumor, lungs, liver, spleen and kidneys were dissected, the tissues were weighed and radioactivity was counted with a a Philips PW 4700 liquid scintillation counter

Tumor growth inhibition

Effect of dose. Mice received a single intravenous injection of an indicated dose of free PLP or liposomal PLP at the time when the tumor became palpable. At 7 days after treatment tumor size was measured and tumor volume calculated according to the formula $V = 0.5 * a^2 * b$, in which a is the smallest and b the largest superficial diameter.

Effect of tumor size. Free PLP or liposomal PLP were i.v. administered at a dose of 20 mg/kg at day 1, 7, and 14 or by single injection at day 7 or day 14 after tumor cell inoculation. As a reference, B16F10 tumors became palpable around 7 days and C26 tumors around 11 days after tumor cell inoculation. Tumor size was measured regularly, and tumor volume was calculated as described above.

Analysis of amount of PLP or prednisolone in tissues

At a tumor volume of approximately 1 cm^3, mice were injected i.v. with 20 mg/kg liposomal PLP or free PLP. At 24 h after injection animals were sacrificed and tumor, liver, and spleen were dissected. The tissues were weighed and homogenized. 2 µg methylprednisolone was added as an internal standard. PLP and free prednisolone were extracted from the tissue with ethylacetate at pH 2, and concentrated under a nitrogen flow. Samples were diluted in ethanol:water 1:1 vol/vol and analyzed by HPLC as described above. Standard lines were prepared by extracting known amounts of PLP and prednisolone from control organs from untreated mice. The detection limit for the HPLC setup was 20 ng/ml

Histology

Tumors were dissected at 72 h after a single i.v. injection of either free PLP or liposomal PLP 20 mg/kg. Tumors were fixed in 10% PBS-buffered formaldehyde and embedded in paraffin. 5 µm slides were cut and stained with hematoxilin/eosin and evaluated by light microscopy.

Statistical analysis

Data were analyzed by one-way ANOVA with Dunnett's post-test using GraphPad InStat version 3.05 for Windows, GraphPad Software (San Diego, USA). Data were logarithmically transformed to correct for significant differences between SD of groups, when appropriate according to Bartlett's test. Spearman rank correlation coefficient was calculated to identify dose-response.

RESULTS

Tissue distribution of LCL

Figure 1 presents tissue distribution data of the LCL at 6 h and 24 h after intravenous injection in C26 or B16-tumor bearing mice. Approximately 60% of the injected dose (ID) was still present in the circulation at 6 h after administration in both mouse models, whereas 15% ID was still circulating at 24 h post-injection. These values correspond to previous data on circulation kinetics of LCL (10). Approximately 7-10% ID could be recovered from tumor tissue in both the C26 and B16F10 model at 24 h after injection, which was approximately two-fold higher than the levels at 6 h post-injection. At both time-points approximately the same amount was present in the livers of both strains of tumor-bearing mice. Relatively low amounts of LCL were recovered from spleen, kidney and lung in the two mouse models both at 6 h and 24 h after injection.

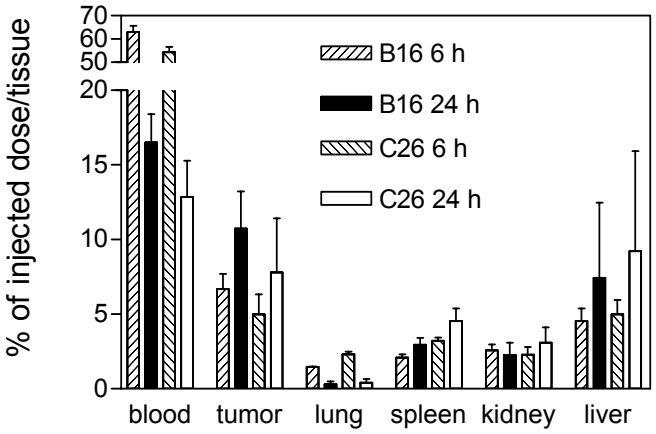

Figure 1. Tissue distribution of 111In-labeled liposomes at 6 h and 24 h after intravenous administration in B16F10-tumor bearing C57Bl/6 mice or C26-tumor bearing Balb/c mice. Tumors weighed approximately 1 g. Mean ± SD, n=5 animals/experimental group.

Anti-tumor activity of liposomal PLP versus free PLP: dose–response relationship

To compare the effect of different doses of liposomal PLP to free PLP on tumor growth, B16 or C26-tumor bearing mice received a single injection of either formulation at the moment that the tumor became palpable. At 1 week after injection the tumor volume was smaller with an inverse relationship to the dose of injected liposomal PLP in both mouse models as shown in Figure 2 (B16: Spearman correlation coefficient $r=-0.92$ ($p<0.001$); C26 Spearman correlation coefficient $r=-0.82$ ($p<0.01$)). 20 mg/kg PLP was the maximum dose that could be administered for the liposomal formulation in view of the maximal injection volume. Treatment of B16 or C26 tumor bearing mice with 20 mg/kg or 50 mg/kg free PLP did not result in significantly different tumor volumes compared to buffer treated control animals (Figure 2).

To evaluate whether the anti-tumor effect was due to a direct cytotoxic effect of prednisolone or PLP on the tumor cells, B16 and C26 cells were incubated *in vitro* with increasing concentrations of prednisolone and PLP. No decrease in cell viability was noted up to the maximum concentration tested of 10 μg/ml (data not shown).

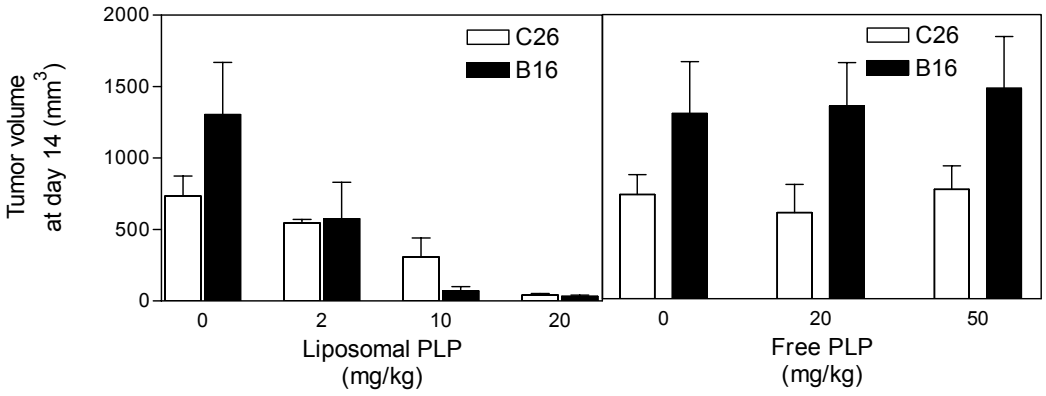

Figure 2. Effect of dose of liposomal (left) or free PLP on tumor growth in B16F10 or C26 bearing mice. Mice received a single injection with the indicated dose and formulation of PLP on the day tumors became palpable (= day 7 after inoculation). Tumor volume after 1 week is reported. Mean ± S.D, n=5 animals/experimental group.

Analysis of level of PLP or prednisolone in tissues

PLP and prednisolone levels at 24 h after i.v. injection of liposomal PLP in liver, spleen and tumor tissue were determined by HPLC analysis. Figure 3 shows that the highest amount of PLP (± 5 µg) was present in the tumor and a similar amount was present in the form of PL. The level of PLP in the spleen was relatively low, whereas the prednisolone level was similar to that in the tumor. Hardly any PLP could be detected in liver tissue, but approximately 20 µg was present in the form of PL. Neither PLP nor prednisolone was detected in any of these tissues at 24 h after injection of free PLP (data not shown).

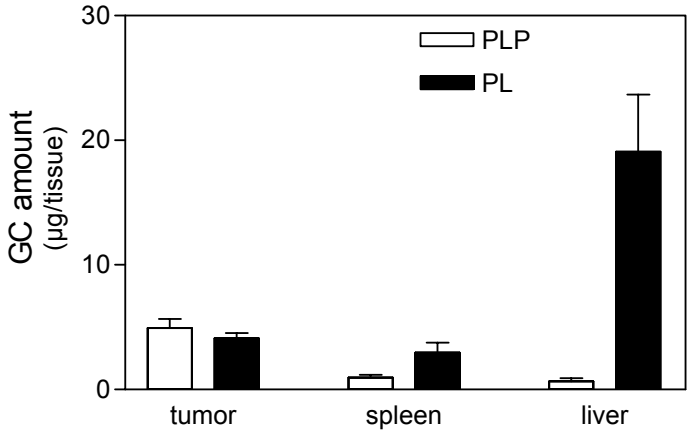

Figure 3. Amount of PLP and PL recovered from mouse tissues. Mice received a single injection of 20 mg/kg liposomal PLP and tissues were excised 24 h later. Mean ± SD, n=5 animals/experimental group.

Dependence of anti-tumor effect on tumor size

To determine if, and to what extent the anti-tumor effect depends on the tumor size at day of treatment, liposomal and free PLP were injected at a dose of 20 mg/kg at day 1, 7, and 14 or a single dose at day 7 or day 14. The results are shown in Figure 4.

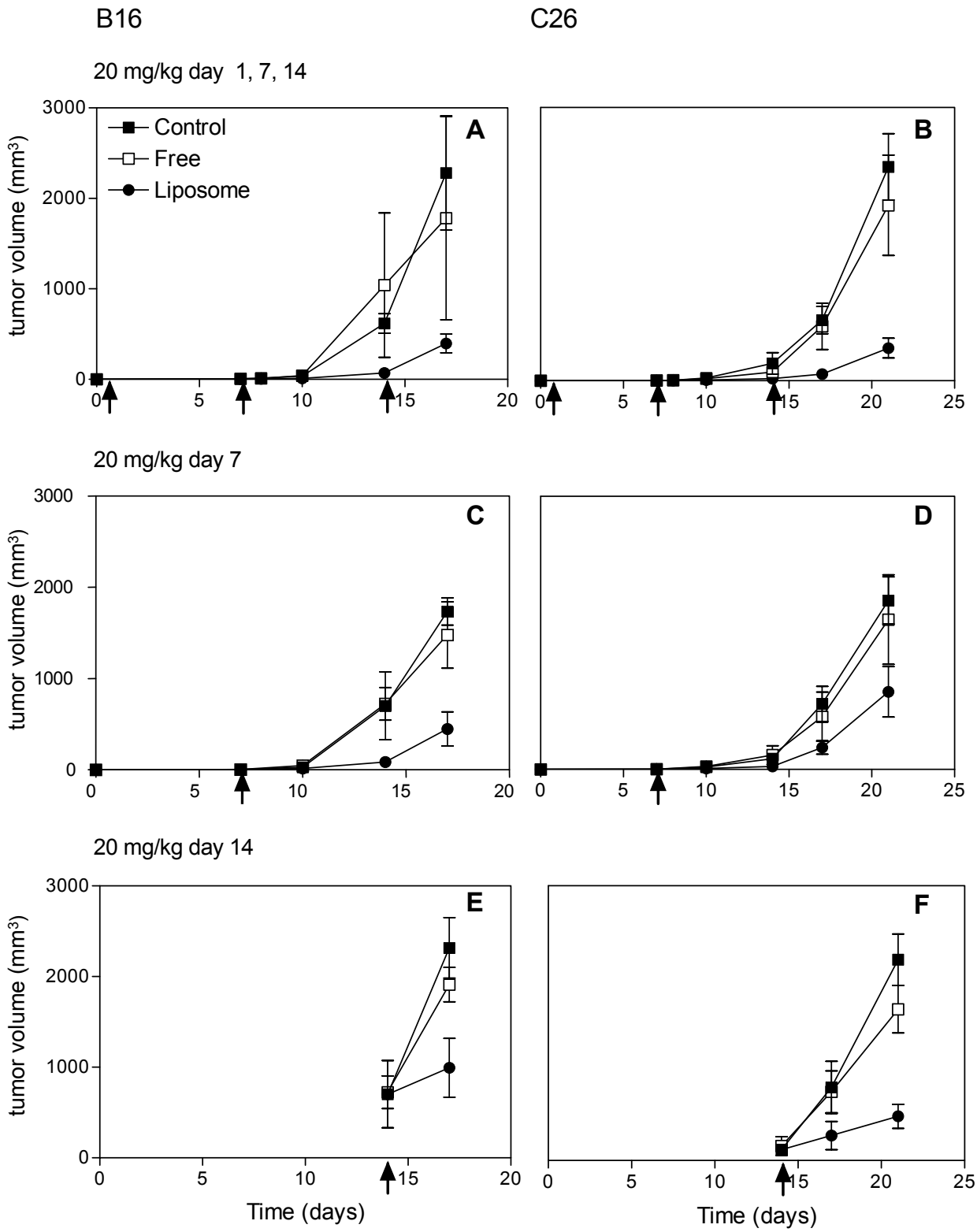

Figure 4. Effect of tumor size on the antitumor effect of free and liposomal PLP. The formulations were injected at a dose of 20 mg/kg at day 1, 7, and 14 (A and B) or single injection at day 7 (C and D) or day 14 (E and F) in B16F10-bearing C57Bl/6 mice (A, C, and E) or C26-bearing Balb/c mice (B, D, and F). Mean ± SD, n=5 animals/experimental group.

B16-model. The tumor volumes of B16-tumor bearing mice that received either no treatment, or treatment with free PLP or liposomal PLP at day 1, 7, and 14 are shown in Figure 4 A. Tumors became palpable at day 7 in all treatment groups indicating that neither of the treatments delayed tumor growth between day 1 and day 7. A second dose of liposomal PLP at day 7 resulted in 92% tumor growth inhibition between day 7 and day 14 as compared to controls ($p<0.05$), whereas free PLP did not affect tumor volume. On day 14, mice received a third injection. At day 17, some of the mice in the free PLP and control group had to euthanized because of large tumor sizes (>2 cm^3), whereas average tumor volume in the liposomal PLP group was approx. 79% smaller ($p<0.01$).

After a single injection of liposomal or free PLP at day 7, a significantly smaller tumor volume was only seen after treatment with liposomal PLP with average inhibition of tumor growth of 89% at day 14 and 67% at day 17 as compared to controls ($p<0.05$, both time-points) (Figure 4 C). A single injection of liposomal PLP at day 14 produced 58% tumor growth inhibition at day 17 compared to controls ($p<0.05$) (Figure 4 E).

C26 model. C26-bearing mice received the first of the three doses of liposomal PLP or free PLP on day 1 and a second on day 7 after tumor cell inoculation. As tumors in all treatment groups became palpable around day 10, the effect on tumor growth of the first injections appeared to be minimal, although tumor volume was 89% smaller in liposomal PLP-treated animals than in controls at day 14. At day 21, 1 week after the third dose at day 14, average tumor volume in liposomal PLP-treated animals was 89% smaller than that in controls ($p<0.01$) (Figure 4 B).

Although a single dose of liposomal PLP on day 7 resulted in 66 % tumor growth inhibition at day 14 and 67% inhibition at day 21, these differences were not statistically significant from the control and free drug-treated groups (Figure 4 D). A single injection of liposomal PLP at day 14 resulted in 78% tumor growth inhibition ($p<0.05$) (Figure 4 F).

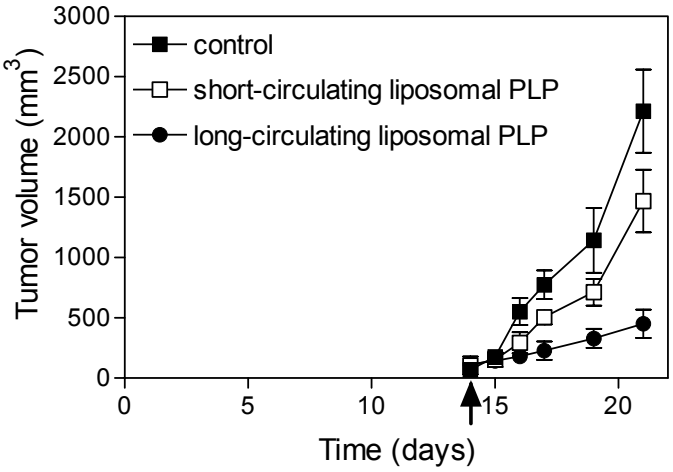

Figure 5. Effect of short-circulating or long-circulating PLP-liposomes on tumor growth. Mice received a single injection of 20 mg/kg of the indicated formulation of PLP on the day tumors became palpable. Mean ± SD, n=5 animals/experimental group.

Importance of the long-circulation property of liposomes for tumor growth inhibition

To determine whether liposomal circulation time is critical for achieving anti-tumor efficacy we tested a SCL and LCL formulation of PLP for anti-tumor activity in C26 tumor-bearing mice. Both formulations were injected at day 14 after tumor cell inoculation in C26-tumor bearing mice. Tumor volume of SCL-encapsulated PLP-treated animals was not significantly different from saline-treated animals, whereas animals treated with LCL-encapsulated PLP experienced a significantly reduced tumor growth rate (Figure 5).

Histological examination of tumor tissue

Histological examination of tumor tissue at 3 days after treatment with a single dose of 20 mg/kg liposomal PLP or free PLP revealed 3 prominent differences between treatment groups as is illustrated in Figure 6 A. Firstly, tumors treated with liposomal PLP were smaller and surrounded by a layer of connective tissue, which was absent in the tumors treated with free PLP. Secondly, liposomal PLP-treated tumors showed areas of apoptotic tumor cells, which were not noted in the free PLP-treated animals. And finally, large fibrin clots were present in some of the larger blood vessels in liposomal PLP-treated tumors, which were not observed in free PLP-treated tumor tissue. A large, leukocyte infiltrated clot is shown in Figure 6 B.

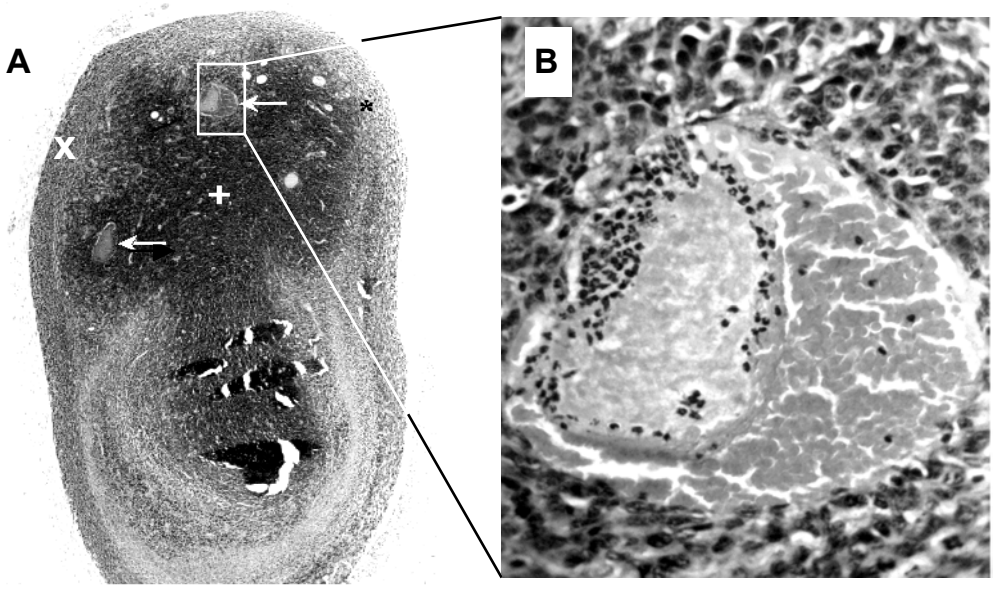

Figure 6. Micrographs of C26 tumor tissue dissected at 3 days after treatment with a single dose of 20 mg/kg of liposomal PLP. Pictures show an overview (A) and an occluded blood vessel (B). Tissues were stained with hematoxilin/eosin. Legend of figure A: **x** layer of connective tissue, **+** apoptotic region, arrows point to occluded blood vessels.

DISCUSSION

The present study demonstrates for the first time anti-tumor effects of LCL-encapsulated GC. Strong anti-tumor effects were observed when liposomal PLP was administered in a low frequency (single dose or weekly) dosing schedule and at substantially lower doses than reported for free GC (conjugates) (5-7).

LCL have been previously used to increase the delivery of a variety of drugs to tumor tissue (16-18). Probably, the best-known formulation in this respect is LCL-encapsulated doxorubicin, marketed as Doxil® or Caelyx® (17). The long-circulating property allows liposomes to extravasate as a result of the enhanced vascular permeability in solid tumor tissue leading to their accumulation at the malignant site (16-18). In both the B16 and C26 tumor models used in this study, 7 to 10% of the injected dose of LCL had localized in the tumor at 24 h after injection, which is similar to previously reported data (10,16-18). At this time-point approximately 15% of the injected dose was still circulating in the blood stream, which is consistent with a long-circulatory behavior.

Furthermore, LCL encapsulation increased levels of the drug in the tumor, liver and spleen at 24 h after injection compared to administration of the free drug. Approximately, 2% of the injected dose of PLP was recovered from the tumor tissue as PLP or prednisolone. This percentage is substantially lower than the 7-10% of the ID of LCL that accumulate at the site of the malignancy. Probably, intratumoral conversion of PLP to prednisolone leads to a redistribution of the drug over the body as prednisolone can easily pass membranes. Conversion of PLP to prednisolone likely forms also the explanation for the virtual absence of PLP in liver tissue.

Administration of LCL-encapsulated PLP resulted in a dose-dependent anti-tumor effect in both the B16 and C26 s.c. tumor model. The maximum dose of 20 mg/kg (determined by the maximal injection volume) resulted in approximately 90% tumor inhibition over a 1 week period, when administered as a single dose at the moment that the tumor became palpable, in both tumor models. Free PLP did not inhibit tumor growth even at a dose of 50 mg/kg in this treatment schedule.

The underlying mechanism of tumor inhibition is at present unclear. Several studies showed that an anti-tumor effect of GC was not directly aimed at the tumor cells, but rather mediated by interference with the tumor vascularization (19-23). Also in the present study PLP or prednisolone did not inhibit the proliferation of the murine tumor cells *in vitro*. It has been suggested that GC's anti-angiogenic effect is mediated by inhibition of endothelial cell proliferation and migration (20) or effects on basement membrane turnover (21). Furthermore, inhibition of production and/or release of pro-angiogenic factors (like plasminogen activator and vascular endothelial growth factor) may play a role (22,23). In addition, the hypothesis that inflammatory processes in and around the tumor are important in the angiogenic cascade suggests that GC's immunosuppressive action may also be of relevance in this respect (24,25). Likely, these mechanisms act together leading to the observed anti-tumor effects.

Regardless of the proposed underlying mechanism, these studies report a concentration-dependent inhibition of the angiogenic process *in vitro* or *in vivo* by GC (19-23). The dose-dependent inhibition of angiogenesis is further illustrated by a study in rats demonstrating that local administration of GC in sponge implants was more effective in inhibiting angiogenesis than systemic treatment (26). Also in the clinical treatment of hemangiomas, intralesional injection of GC is often used (27). The importance of prolonged high local drug levels is further supported by the observation in this study that the same dose of liposomal PLP in SCL inhibited tumor growth to a much lower extent. SCL are rapidly taken up by macrophages mainly in liver and spleen and are therefore unable to accumulate at the tumor. Consequently, the limited localization of SCL in the tumor is paralleled by a decrease in activity.

The lack of an inhibitory effect of liposomal PLP against small, not-yet palpable, tumors as observed in this study, is indicative of the importance of tumor localization of the liposomes for anti-tumor efficacy. Both in the B16 and C26 model, liposomal PLP injection at 1 day after tumor cell inoculation did not delay the time-point at which the tumor became palpable. At 24 h after tumor cell inoculation, the tumor mass is still minimal and vascular integrity is hardly affected yet. Therefore the circulating liposome particles are not able to extravasate at this time point. Once a tumor mass has formed, however, administration of liposomal PLP reduced tumor growth rate significantly, irrespective of the size of the tumor at the time of liposome injection.

Histological evaluation indicates that apoptosis of tumor cells in the core of the tumor occurs, which could be the result of inhibition of angiogenesis as the cells at this location are most dependent on intratumoral blood supply. Another observation, which was made solely in some of the liposomal PLP treated tumors, was the presence of blood clots inside the larger blood vessels in the tumor tissue. As thrombosis has been suggested to precede angiogenesis, we may be dealing with tissue that is again in or converting to a pro-angiogenic state (28,29).

In conclusion, the present study shows for the first time potent anti-tumor efficacy of LCL-encapsulated PLP. This formulation yields high intratumoral levels of PLP for a prolonged period of time. The advantage that the current system may offer for clinical use is the relatively low dose and low frequency schedule with which PLP needs to be administered to produce anti-tumor efficacy.

ACKNOWLEDGEMENT

Dr. G.A. Koning is gratefully acknowledged for critical reading of the manuscript.

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CHAPTER 7

THERAPEUTIC INDEX OF GLUCOCORTICOIDS CAN BE OPTIMIZED BY ENCAPSULATING HIGH-CLEARANCE GLUCOCORTICOIDS IN LONG-CIRCULATING LIPOSOMES

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ABSTRACT

Long-circulating liposomes (LCL) may be useful as drug-targeting vehicles for anti-inflammatory agents in arthritis, since they selectively home at inflamed joints after i.v. administration. Previously we showed in experimental arthritis that encapsulation of glucocorticoids (GC) as water-soluble phosphate esters in PEG-liposomes resulted in a strong improvement of the anti-inflammatory effect as compared to the free drug. In the present study, we compared the therapeutic activity and adverse effects induced by 3 different GC encapsulated in LCL in an attempt to further optimize the therapeutic index of liposomal GC in arthritis. Our data show that with GC (dexamethasone, budesonide) of higher potency than prednisolone, the therapeutic activity of liposomal GC can be increased. However, side effects at the level of body weight and hyperglycemia were noted, related to the sustained free GC level observed after injection of the liposomal GC. An inverse relationship with the clearance rate of the GC in question was shown. This study stresses the importance of a high clearance rate of the GC to be encapsulated for achieving a maximal therapeutic index with liposomal GC. Therefore high-clearance GC, which until now are only applied in local treatment approaches, may be very useful for the development of novel, highly effective anti-inflammatory preparations for systemic treatment of inflammatory disorders.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder, involving joint inflammation and progressive cartilage destruction (1). Glucocorticoids (GC) are highly effective anti-inflammatory drugs but their use in arthritis therapy is controversial due to a high incidence of serious adverse effects occurring during chronic treatment (2-4). As a result of rapid elimination from the circulation and unfavorable tissue distribution, systemic treatment with GC results in poor target localization of the drug, which often necessitates the use of high doses and intensive dosing schedules (5,6). Targeted delivery of GC can greatly increase the concentration of the drug in the inflamed joints and therefore a less intensive dosing regimen may be sufficient for an adequate therapeutic response with minimal risk for side effects.

Long-circulating liposomes have been extensively studied as targeted drug carrier systems in oncology and infectious diseases. They have been shown to selectively accumulate at the corresponding sites of pathology (7-10). The phenomenon of selective targeting to pathological sites can be attributed to locally enhanced permeability of the vascular endothelium, allowing small-sized liposomes to extravasate and accumulate in the extravascular tissue (11,12).

In a previous study we showed that a single i.v. injection of GC in long-circulating liposomes yielded a rapid, complete and durable disease remission in a rat model of adjuvant arthritis (AA). We selected prednisolone as model GC and encapsulated the water-soluble inactive phosphate ester prednisolone phosphate (PLP) to achieve a stable liposome formulation as PLP in free form is quickly converted into active prednisolone in blood or tissues. Intensive treatment with repeated daily injections of free PLP could by far not match the effect of a single dose of the liposomal drug (see Chapter 3). Pharmacokinetic analysis of blood samples taken after administration of liposome-encapsulated PLP revealed not only the presence of liposome-bound PLP but also a low, sustained level of unencapsulated prednisolone in the circulation. The low, sustained level of free prednisolone did not contribute to the therapeutic activity of liposomal PLP.

A drawback for the translation of therapeutic effects observed in rat models, to humans is that the pharmacokinetic behavior of prednisolone in rats is strongly different from that in human beings. Therefore, in the present study, we selected dexamethasone phosphate (DXP) for incorporation in liposomes, as the pharmacokinetics of dexamethasone in rats is similar to its pharmacokinetics in humans.

First, we investigated whether incorporation of DXP in liposomes is also therapeutically beneficial in the AA model. In view of the stronger potency of dexamethasone over prednisolone higher therapeutic efficacy was anticipated.

Second, it was evaluated whether the sustained free drug level observed after i.v. administration of liposomal GC is related to the occurrence of systemic side effects. As parameters for systemic side effects, treatment-induced loss of body weight and increase of blood glucose concentration were measured as these parameters can be quickly, frequently

and accurately be measured (as shown by Kaur et al., 1989 (13) and Ogawa et al., 1992 (14), respectively).

Third, it was investigated whether the sustained free drug level correlates with the clearance rate of the free GC that appears in the circulation after injection of the corresponding liposomal formulation. If the clearance rate is an important determinant of the free drug level, the chance for side effects may be minimized by encapsulation of high-clearance GC. We compared PEG-liposomes with dexamethasone (relatively slow clearance rate) to PEG-liposomes containing GC with a higher clearance rate (prednisolone, budesonide), with respect to pharmacokinetics, therapeutic response in the AA model, and systemic side effects, such as body weight loss and increased blood glucose values. With budesonide as an example, the results suggest that topical high-clearance GC are interesting candidates for encapsulation in long-circulating liposomes and application in the treatment of arthritis. This study is the first to report the use of topical high-clearance GC in a systemic drug-targeting approach.

MATERIALS AND METHODS

Preparation of liposomal GC

Basically, the same long-circulating poly(ethylene glycol) (PEG) liposomes were used as in previous studies, in which was shown that this type of liposomes can selectively accumulate in inflamed joints and induce strong local anti-inflammatory activity (Chapter 3 and 4). The liposomes were prepared by the film-extrusion method (15). Briefly a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma Chemical Co., Poole, UK) and distearoyl phosphatidylethanolamine-PEG 2000 (PEG-DSPE) (Avanti Polar Lipids, Alabaster, AL, USA) in a molar ratio of 1.85 : 1.0 : 0.15 respectively. The lipid solution was transferred to a round-bottom flask and a lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml of prednisolone disodium phosphate (PLP), dexamethasone disodium phosphate (DXP) (both obtained from Bufa, Uitgeest, The Netherlands) or budesonide disodium phosphate (BUP) (synthesized by Syncom, Groningen, The Netherlands) dissolved in sterile water. The resulting lipid dispersion was sized to a diameter between 90 and 100 nm by multiple extrusions through polycarbonate filter membranes. The unencapsulated GC-phosphate was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). The mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). The phospholipid content was determined with a phosphate assay (16) in the organic phase after extraction of liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the GC-phosphate content by high performance liquid chromatography with a mobile phase of acetonitril-water with a pH of 2, followed by UV-detection at 254 nm. The liposomal preparations contained between 3.5 and 4.5 mg GC-phosphate and an average of 60 µmol phospholipid/ml.

Rat adjuvant arthritis

The Dutch Committee of Animal Experiments approved all animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (170-200 g) were obtained from Maastricht University, Maastricht, the Netherlands. To induce arthritis, 100 ml of incomplete Freund's adjuvant (IFA) containing 10 mg/ml of heat-inactivated Mycobacterium tuberculosis (Mt) (both purchased from DIFCO laboratories, Detroit, MI, USA) was injected intracutaneously at the base of the tail (17). At day 10 after the immunization, the first signs of joint inflammation became visible, together with a loss of body weight as a result of the disease. 20 days post-immunization the disease reached maximal severity, after which the inflammation process gradually resolved. Starting at day 10, the rats were daily examined for the visual signs of inflammation and the disease-induced weight drop. The severity of the joint inflammation was graded by assigning a score to each paw from 0 to 4, based on erythema, swelling and deformation of the joints. The sum of these four grades for each animal is the clinical score

and can vary from zero up to 16. Besides the development of paw inflammation, the disease results in a loss of body weight that can easily be monitored by daily weighing of the rats.

Therapeutic activity

All rats were treated on day 14 or 15 post-immunization, when the average score of all rats in the experiment reaches 7, which was approximately half the maximal scores reached in these experiments. At the day of treatment, groups of five rats were formed with equal average clinical scores. All preparations were given intravenously in the tail vein. As the pharmacokinetics of PEG-liposomes have been shown to be lipid dose-independent, the administered dose of phospholipid was allowed to vary with the different liposomal GC preparations (18). When multiple injections of free GC were required, each following day treatment was repeated at the same time. The effect of treatment on clinical scores and body weight loss was monitored daily from day 10 until day 30 post-immunization. Control rats were treated with 150 μmol total lipid/kg empty PEG-liposomes.

Systemic adverse effects

As parameters for systemic activity loss of body weight and increase in blood glucose concentrations were evaluated. Loss of body weight is a phenomenon that is generally observed upon systemic GC treatment in rats (13). In this study the loss of body weight because of treatment with GC is clearly additional to the weight loss resulting from the induction of experimental arthritis. Besides the induction of body weight loss, systemic GC treatment can induce hyperglycemia (14). Monitoring the increase of blood glucose was performed by using a blood glucose meter (EuroFlash, LifeScan Inc, Miltiplas, USA).

Determination of liposomal GC-phosphate and free GC in the circulation

In a previous study we showed that PLP remained stably entrapped in PEG-liposomes upon i.v. injection (Chapter 3), since at different time-points post-injection PLP was detected in the same quantities as a liposome bilayer marker (assuming that unencapsulated PLP is quickly and completely converted to prednisolone after entering the circulation). To evaluate the stability of the other liposomally encapsulated GC-phosphates (DXP and BUP), plasma concentration-time curves of the different liposomal GC-phosphates were measured after injection of a dose of 10 mg/kg in healthy rats and were compared to PLP-PEG-liposomes. Concentrations of liposomal GC-phosphates were determined by plasma extraction followed by HPLC-determination (19). Concentrations as low as 200 ng/ml could be measured accurately. Quantities of free GC after injection of 10 mg/kg liposomal GC-phosphate in healthy rats could simultaneously be detected with the same assay in one single run with the phosphate ester. Concentrations of free GC in the extracts could be determined accurately down to a concentration of 50 ng/ml.

Statistical analysis

For statistically assessing and comparing therapeutic efficacy in different groups the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups regarding other parameters, a one-way analysis of variance or a Students T-test was performed. P-values of less than 0.05 were considered significant.

RESULTS

Therapeutic activity in rat AA: DXP-PEG-liposomes vs. free DXP

Figure 1 shows the anti-inflammatory effect of 2 mg/kg dexamethasone phosphate (DXP) i.v. in free form and encapsulated in PEG-liposomes. A single dose of 2 mg/kg free DXP significantly suppressed paw inflammation during three days. The same dose encapsulated in PEG-liposomes resulted in complete disappearance of the clinical signs of AA within two days. Complete remission of the disease symptoms lasted until day 20 (6 days post-treatment) after which joint inflammation gradually reappeared, reaching the inflammation score of the saline control group around day 24. The same therapeutic response could be realized by 5 daily injections of 2 mg/kg free DXP.

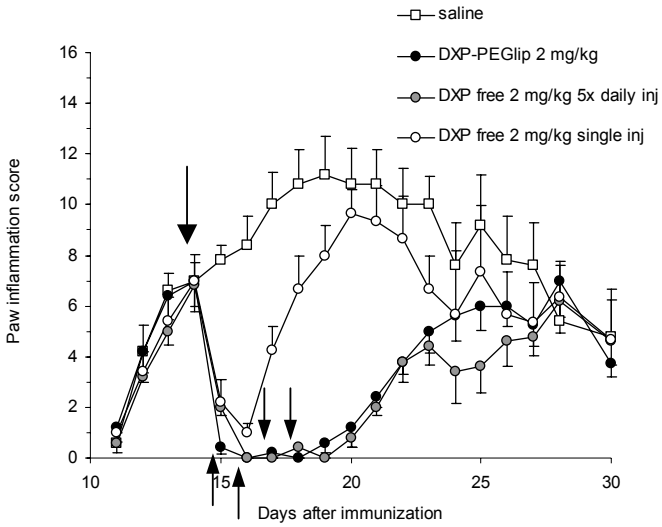

Figure 1. Therapeutic activity in rat AA of 2 mg/kg DXP-PEG-liposomes versus 2 mg/kg free DXP given as single or multiple daily treatment. Means of 5 rats are shown. Vertical bars show SEM. Arrow indicates first day of treatment.

Adverse effects: DXP-PEG-liposomes vs. free DXP

Figure 2 A shows the effect of the treatment on the total body weight of AA rats. Both liposomal DXP as well as single and multiple treatment with free DXP resulted in treatment-induced weight loss additional to the body weight loss as a result of the disease (saline treatment). Although equally effective at a therapeutic level, repeated administration of 2 mg/kg free DXP generated a stronger treatment-induced loss of body weight than a single injection of 2 mg/kg liposomal DXP ($p < 0.05$ at day 18 post-immunization). In Figure 2 B it is shown that 2 mg/kg DXP in liposomal and in free form (single and repeated injections) enhanced blood glucose levels to a similar extent during the first days after treatment.

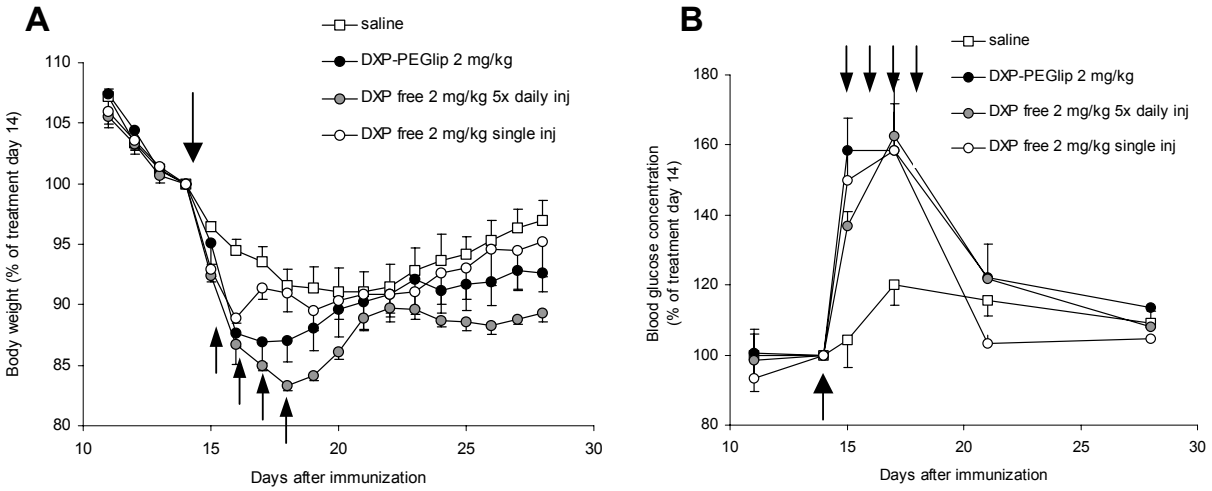

Figure 2. Systemic effects as a result of 2 mg/kg unencapsulated DXP (single and multiple dose), and 2 mg/kg DXP-PEG-liposomes. (A) Effect on total body weight. Vertical bars show SEM. (B) GC-induced hyperglycemia. The percentage of the blood glucose concentration at day of treatment is shown. Vertical bars show SD. In both A. and B. means of 5 rats are shown. Arrows indicate treatment days.

Therapeutic activity and adverse effects: PLP-PEG-liposomes vs. DXP-PEG-liposomes

To investigate the role of clearance rate in the therapeutic index of liposomal GC, prednisolone phosphate (PLP) was encapsulated, as prednisolone is a GC, which has in rats a much higher clearance rate than dexamethasone. However, as these two GC are known to differ in potency, first the therapeutic activity of liposomal PLP was compared with liposomal DXP. Figure 3 A shows the comparative effect of a single dose of 2 mg/kg and 10 mg/kg of PLP-PEG-liposomes and DXP-PEG-liposomes on rat adjuvant arthritis scores. A clear dose-response relation is observed with both liposomal GC. A dose of 10 mg/kg PLP-PEG-liposomes was equally effective as 2 mg/kg DXP-PEG-liposomes, indicating that liposomal PLP is approximately 5 times less potent than liposomal DXP.

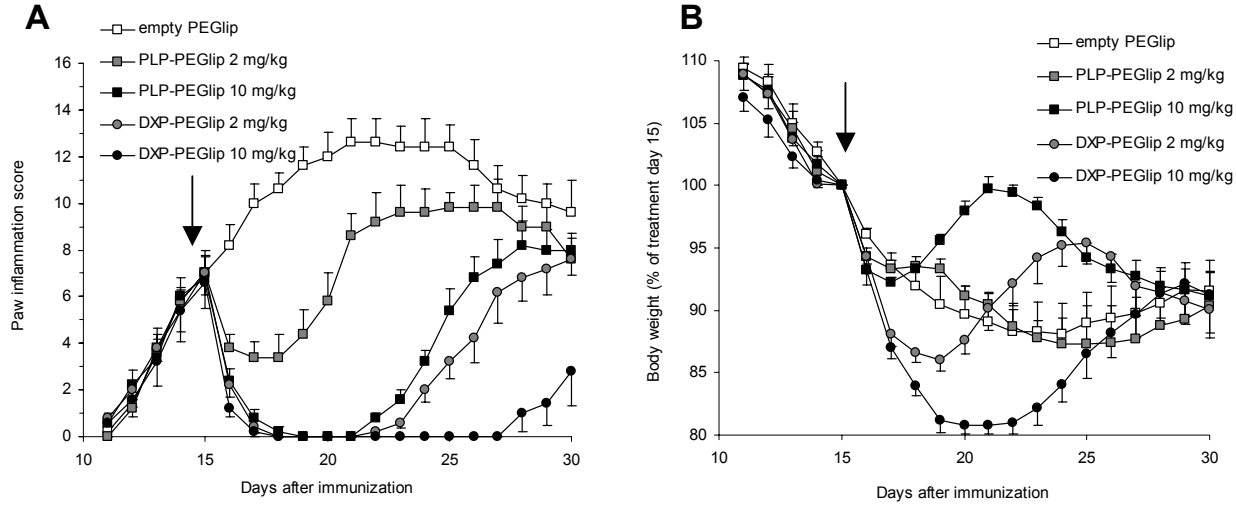

Figure 3. Relative potency of PLP-PEG-liposomes vs. DXP-PEG-liposomes. (A) Effect on joint inflammation in rat adjuvant arthritis, and (B) effect on body weight. Body weight is shown as percentage of the body weight at day of treatment. Means of 5 rats are shown. Vertical bars show SEM. Arrow indicates treatment (day 15).

Figure 3 B shows that 10 mg/kg PLP-PEG-liposomes reversed the disease-induced process of body weight loss between day 2 and 7 post-treatment. A dose of 2 mg/kg DXP-PEG-liposomes produced a similar response, however, body weight gain started 2 days later, between day 19 and day 24. In the period between day of treatment (day 15) and body weight gain, an additional loss of body weight was observed with liposomal DXP, which was not significant with liposomal PLP. Rats treated wit 2 mg/kg liposomal DXP showed an additional weight loss of up to 5.5% as compared to rats in the control group over a period of 4 days before body weight gain was observed. With 10 mg/kg liposomal DXP, this additional body weight loss even reached 9.2%, lasting for more than a week.

Therapeutic activity and adverse effects: BUP-PEG-liposomes vs. DXP-PEG-liposomes

The anti-inflammatory effect of a dose of 1 mg/kg BUP-PEG-liposomes was compared to the effect of 1 mg/kg and 2 mg/kg DXP-PEG-liposomes in the rat AA model (Figure 4). Both liposomal DXP and liposomal BUP are highly effective in AA, causing a complete remission of joint inflammation at a 10-fold lower dose as compared to liposomal PLP. Importantly, Figure 5 B shows that 1 mg/kg liposomal BUP induces an almost complete regain of the disease-induced loss of body weight as a result of its therapeutic effect. However, the opposite is observed after both 1 mg/kg and 2 mg/kg liposomal DXP, which induced an additional treatment-induced body weight loss. The reversal of disease-induced body weight loss as a result of the therapeutic effect was also seen with liposomal DXP, but this was occurring after the period of additional treatment-induced body weight loss.

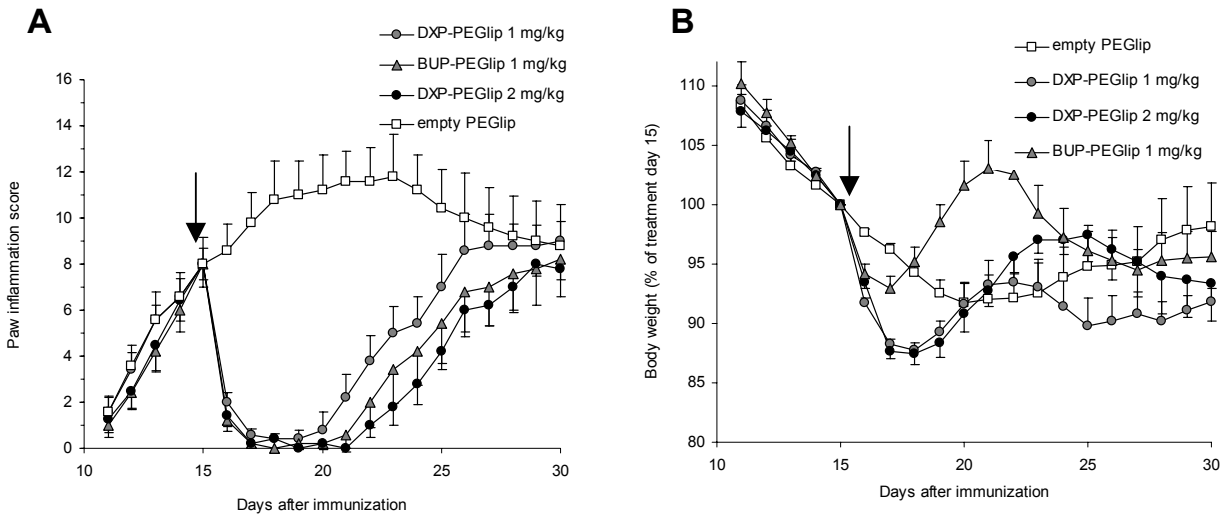

Figure 4. Relative potency and systemic activity of BUP-PEG-liposomes vs. DXP-PEG-liposomes. (A)Effect on joint inflammation, and (B) effect on body weight of 1 mg/kg liposomal BUP and 1 and 22 mg/kg liposomal DXP. Body weight is shown as percentage of the body weight at day of treatment. Means of 5 rats are shown. Vertical bars show SEM. Arrow indicates treatment (day 15)

Plasma concentrations: liposomal GC vs. free GC

Figure 5 A shows the plasma concentration-time profile of the three different GC-phosphates: PLP, DXP and BUP, after injection of a dose of 10 mg/kg encapsulated in PEG-liposomes. All three liposomal GC follow the same plasma concentration-time profile. As in a previous study PEG-liposomal PLP was shown to be completely contained within the liposome particles in the circulation, based on these data, complete retention in the liposomes may also be expected with both DXP and BUP in PEG-liposomes.

Despite equal dose and identical plasma concentration-time profile of the three liposomal GC-phosphates, strong differences are observed regarding the plasma concentration-time profile of the free (i.e. not bound to liposomes) parent drug detected in the circulation after treatment with the liposomal formulations. Treatment with liposomal DXP yielded the highest free drug levels, whereas treatment with liposomal BUP and PLP resulted in similar, but much lower levels of free GC. Roughly, the areas under the plasma concentration-time curves of free GC in the circulation appeared to be inversely correlated with the reported clearance values (in rats: approx. 0.2 $L \cdot h^{-1} kg^{-1}$ for dexamethasone (20), 1.5 $L \cdot h^{-1} kg^{-1}$ for budesonide (21) and 2.3 $L \cdot h^{-1} kg^{-1}$ for prednisolone (22)).

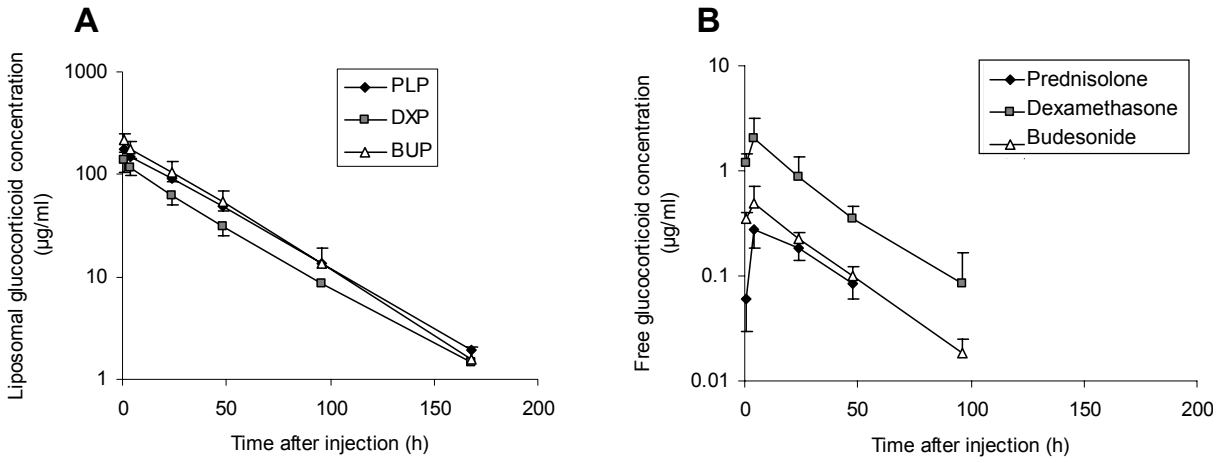

Figure 5. Plasma concentrations of liposomal glucocorticoid phosphate (A) and released free glucocorticoid (B) in the circulation upon injection of 10 mg/kg glucocorticoid phosphate-PEG-liposomes. Data represent means of 4 rats, Vertical bars show SD.

DISCUSSION

In previous studies we showed that long-circulating liposomes extravasate into inflamed joints in experimental rat and murine models of arthritis. Liposomal encapsulation of PLP enhanced the local anti-inflammatory activity to such an extent that even a single i.v. injection of 10 mg/kg liposomal PLP yielded complete, rapid and long-lasting remission of joint-inflammation. In the present study, we compared the therapeutic activity and adverse effects of 3 different GC, encapsulated in water-soluble phosphate form in long-circulating liposomes in an attempt to further optimize the therapeutic index of liposomal GC in arthritis. However, increased efficacy is only clinically relevant when the adverse effects are not increased such that the therapeutic index of the drug is not improved upon liposomal encapsulation. In the present study we focus on possible adverse effects of liposomal GC and evaluate how the therapeutic index of liposomal GC can be optimized.

Dexamethasone was chosen as model GC, as its pharmacokinetics in rats are quite similar to that in humans. As the potency of dexamethasone is higher than that of prednisolone, which was used in our previous study, the first objective was to find the dose at which liposomal dexamethasone phosphate (DXP) induced complete remission of joint inflammation. In rat AA, it appears that a single i.v. injection of 2 mg/kg liposomal DXP can induce a full disease remission for almost a week. This therapeutic response roughly equals the previously observed response in AA of 10 mg/kg liposomal PLP (Chapter 3), indicating that liposomal DXP is approximately 5 times more potent than liposomal PLP. Therapeutic benefit could also be realized with free DXP. However, 5 daily injections of 2 mg/kg were required to produce the same response as a single treatment with 2 mg/kg liposomal DXP, indicating that liposomal encapsulation strongly enhances the therapeutic activity of the drug (Figure 1).

The second objective of this study was to evaluate possible systemic side effects of induced by sustained level of free GC in the circulation after injection of liposomal GC. Such levels were observed in our first study with liposomal PLP. We showed that these levels were not contributing to the increased therapeutic effect. However, they may contribute to the induction of systemic adverse effects. First, the effect on body weight was evaluated. Besides paw inflammation, induction of AA in rats generally leads to a gradual fall of body weight. Therapeutic activity in the model is not only detectable by reversal of paw inflammation, but also by reversal of disease-induced body weight fall (17). Reversal of body weight fall was clearly observed in our previous study with PLP-PEG-liposomes. In the present study, however, instead of a reversal, liposomal DXP induced an extra drop in body weight occurring during the first five days after treatment (Figure 2 A). This treatment-induced body weight loss was additional to the disease-induced body weight drop and could be reproduced in healthy rats (data not shown). Body weight loss as a result of i.v. GC has been earlier reported for rats (13) and can be considered as a relevant parameter for systemic adverse events. As in our study the treatment-induced body weight fall was also seen with

free GC, it is likely that liposomal DXP induced this adverse effect as a result of the presence of free dexamethasone in the circulation.

Besides the effect on body weight also the effect on blood glucose levels of GC can be used as a parameter for systemic activity (14). In our study, monitoring blood glucose levels during the course of the disease showed that both liposomal DXP and free DXP caused a limited, but significant hyperglycemia during the first days after treatment (Figure 2 B). This observation again points to the presence of free dexamethasone in the systemic circulation after injection of liposomal DXP. Interestingly, an equipotent dose of liposomal PLP did not result in significant systemic adverse effects. Instead of a treatment-induced body weight loss, a strong regain of body weight was revealed in the first week after treatment with 10 mg/kg liposomal PLP, which clearly corresponded with the remission of paw inflammation (Figure 3). Furthermore, no significant rise of blood glucose concentration was revealed upon 10 mg/kg liposomal PLP (data not shown). These observations suggest that the fraction of the i.v. administered dose of liposomal GC-phosphate that becomes available in the circulation as free GC may be much lower with liposomal PLP than with liposomal DXP.

The third objective of the study was to evaluate whether there is a relation between the clearance rate of the encapsulated GC-phosphate ester and the quantity of free parent drug that becomes available in the circulation after i.v. administration of liposome-encapsulated GC-phosphate. In our previous study injection of liposomal PLP yielded detectable levels of free prednisolone in the circulation. As the liposome particles appeared not to leak PLP in the circulation, it was suggested that in particular phagocytes in liver and spleen release the encapsulated GC-phosphate after uptake and degradation of the liposomes (23,24), after which the phosphate ester is converted into active GC. With a higher clearance rate of the free drug, one would expect the quantity of GC present in the circulation in free form to be less, as clearance rate and area under the plasma concentration-time curve of drug in the circulation are inversely related. In rats, there is a clear difference between prednisolone and dexamethasone regarding their clearance rate. The clearance rate of prednisolone is reported to be approximately 2.3 $L \cdot h^{-1} kg^{-1}$ (21), whereas for dexamethasone the clearance rate is not higher than 0.2 $L \cdot h^{-1} kg^{-1}$ (19,25). Therefore, the absence of systemic activity of liposomal PLP regarding body weight loss (Figure 3b) and hyperglycemia may indeed be explained by its high clearance rate in rats.

Our results show that liposomal encapsulation of PLP results in a stronger improvement of the therapeutic index than liposomal encapsulation of DXP. However, this observation may only apply to the rat. In humans, the clearance rate of prednisolone from the circulation is quite similar to that of dexamethasone. To optimize the therapeutic index of i.v. liposomal GC in RA patients, other GC should be selected with high clearance rates after i.v. administration in humans without forming active metabolites. Such may be found among the GC that are used for local treatment of asthma and rhinitis (26,27). From this group we selected budesonide, one of the GC reported to have a high clearance rate forming metabolites that are almost completely devoid of systemic activity (28). To successfully encapsulate budesonide in PEG-liposomes, the water-soluble phosphate ester was

synthesized (BUP), a derivative, which has not yet been commercialized. The results show that the liposomal form of this novel budesonide derivative is at least as effective as liposomal DXP in rat AA (Figure 4 A) while showing less systemic side effect (Figure 4 B).

Comparing the plasma concentration-time curves of liposomal BUP with liposomal DXP and liposomal PLP after i.v. injection of equal doses revealed identical profiles for all three liposomal GC. As we showed before that no leakage of PLP from PEG-liposomes occurred, such may also be assumed for liposomal DXP and liposomal BUP. In contrast, the sustained free drug levels after injection of the three liposomal GC formulations greatly differed from each other with an inverse relationship with the clearance rate of the GC in question (Figure 5 B). The observation that free budesonide levels were slightly higher than free prednisolone is in agreement with the slightly lower clearance rate of budesonide reported in rats (1.5 $L \cdot h^{-1} kg^{-1}$ as compared to 2.3 $L \cdot h^{-1} kg^{-1}$ for prednisolone) (20,21). However, this does not reflect the human situation, as in humans the clearance rate of prednisolone is much lower than that of budesonide (29,30).

In conclusion, our data show that with the use of GC (dexamethasone, budesonide) of higher potency than prednisolone, the therapeutic activity of liposomal GC can be increased. However, sustained free GC levels were observed after injection of the 3 liposomal GC-phosphates, which showed an inverse relationship with the clearance rate of the GC used. As the sustained free GC levels can cause systemic side effects, this study stresses the importance of a high clearance rate of the free GC in question for achieving a maximal therapeutic index with liposomal GC. Therefore high-clearance GC, which until now are only applied in local treatment approaches, may be very useful for the development of novel, highly effective anti-inflammatory preparations for systemic treatment of inflammatory disorders.

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CHAPTER 8

COMPLEMENT ACTIVATION - RELATED HYPERSENSITIVITY REACTIONS CAUSED BY PEGYLATED LIPOSOMES

SEARCH FOR AN ALTERNATIVE LONG-CIRCULATING LIPOSOME FORMULATION WITHOUT COMPLEMENT ACTIVATION

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ABSTRACT

PEGylated long-circulating liposomes have been reported to cause immediate hypersensitivity reactions in 5-10% of the patients treated. These reactions may be explained by liposome-induced complement activation. The aim of this study was to design a long-circulating liposome formulation that does not induce complement activation. We monitored the formation of complement terminal complex SC5b-9 *in vitro* in human serum samples as well as hemodynamic changes in pigs upon i.v. injection of several PEGylated and non-PEGylated liposome formulations.

It was found that Doxil® (PEG-liposomal doxorubicin), similar PEG-liposomes without drug as well as size-matched (90 nm) empty non-PEG-liposomes, all caused significant *in vitro* complement activation as well as hypersensitivity reactions in pigs, indicating that PEG-PE and/or its negative charge cannot be the sole underlying cause. Smaller (<70 nm) non-PEG-liposomes composed of DSPC and cholesterol caused no complement activation and completely lacked the induction of hemodynamic changes in pigs, suggesting that size is an additional major factor in complement activation responses.

To evaluate the usefulness of these small non-PEG-PEGylated DSPC-cholesterol liposomes for passive targeting purposes, dexamethasone phosphate was encapsulated and this liposomal formulation was given to rats with experimental arthritis. Interestingly, our data show that these liposomes can circulate at least as long as PEG-liposomes. The therapeutic activity of the liposomally encapsulated drug was similar for the PEG-liposomes and non-PEG-liposomes in this study, indicating that small-sized (<70 nm) non-PEGylated liposomes may be preferred over PEG-liposomes as carrier for passive drug targeting purposes, as they appear not to induce complement activation.

INTRODUCTION

PEG-liposomes have extensively been studied as potential carriers for targeted drug delivery to tumors, infections and sites of inflammation, as they have been shown to selectively accumulate at these sites (1-4). The success of PEG-liposomes in the field of oncology has led to a growing use of this formulation in the clinic. One preparation is currently on the market for the treatment of solid tumors: Doxil® (Caelyx® in Europe), a PEGylated long-circulating liposome (LCL) formulation of doxorubicin, while others are in clinical trials.

The therapeutic value of PEG-liposomes in infection has been shown in a range of preclinical studies (5). Evaluation in experimental models of infection revealed that the therapeutic activity of antibacterial drugs can be improved by encapsulation in PEG-liposomes. In rat and murine models of experimental arthritis encapsulation in PEG-liposomes strongly improved the anti-inflammatory activity of glucocorticoids (see Chapter 3 and 4). Such promising results warrant clinical studies in patients in a short term.

However, PEG-liposomal formulations such as Doxil® can cause immediate allergic reactions in patients. These reactions have been observed in a significant proportion of patients. The symptoms occur upon the first infusion and include dyspnea, tachypnea, facial swelling, headache, chills, hypo- and/or hypertension, chest pain and back pain (6-9). They are generally mild and quickly resolve after interruption and resumption of the infusion at a slower rate. Most patients can receive further infusions without any complications. The reactions have been dubbed “pseudoallergic”, as no prior sensitization is needed to induce them. Clinical studies performed with radioactively labeled PEG-liposomes without drug for diagnostic purposes also revealed pseudoallergic reactions, supporting the suggestion that the observed effects are due to the liposomal carrier rather than the drug (10).

In recent reports it was hypothesized that PEGylated liposome formulations induced these phenomena as a result of complement activation (11,12). A key argument for this hypothesis came from experiments in a porcine model of liposome-induced cardiopulmonary distress (13,14), which showed that the PEG-liposome-induced hemodynamic reactions in pigs are a consequence of complement activation (15). A relationship between complement activation and pseudoallergic reactions was recently confirmed in a clinical study in patients treated with Doxil® (16). Possibly the use of PEG-phosphatidyl ethanolamine (PEG-PE) is a key factor in the induction of complement activation.

The ultimate aim of this study was to design LCL, which do not induce complement activation. To this end, different types of PEGylated and non-PEGylated liposomes were evaluated regarding complement activation in human serum, pseudoallergic reactions in pigs, and long-circulating properties and therapeutic benefit in a rat model of experimental arthritis. For the two latter purposes, the anti-inflammatory glucocorticoid dexamethasone phosphate (DXP) was encapsulated as a model drug, as a previous study revealed that encapsulation of glucocorticoids in PEG-liposomes, resulted in a strong increase of the therapeutic benefit of the glucocorticoid in question by selective targeting to inflamed joints (Chapter 3).

MATERIALS AND METHODS

Liposomes

Liposomes were prepared by the film-extrusion method (17). Briefly, a lipid solution was prepared in ethanol, containing the phospholipid dipalmitoyl phosphatidylcholine (DPPC) or distearoyl phosphatidylcholine (DSPC) (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 2:1. For PEG-liposomes, 7.5 mole % of the amount of total lipid in the mixture of poly(ethylene glycol)2000 coupled to distearoyl phosphatidylethanolamine (PEG-DSPE) (Avanti Polar Lipids, Alabaster, AL, USA) was added. The lipid solution was transferred to a round-bottom flask and a lipid film was created by rotary evaporation. The film was hydrated with 0.9% saline buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH: 7.4) in sterile water. DXP was encapsulated by dissolving 100 mg/ml in the hydration buffer before hydration. The resulting lipid dispersion was sized to the desired diameter by multiple extrusions through polycarbonate filter membranes. Non-encapsulated DXP was removed by dialysis with Slyde-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Zeta potentials were measured with a Zetasizer (Malvern Ltd., Malvern, UK). Phospholipid content of the organic phase after extraction of liposomal preparations with chloroform was determined with a phosphate assay (18). DXP was determined by a reversed phase HPLC assay in the aqueous fraction after extraction.

In vitro complement activation assay in human serum samples

Blood samples from healthy volunteers were collected and serum was prepared and stored (at –20 °C) as described earlier (19). Complement activation was assessed by ELISA kits (Quidel Co., San Diego, CA, USA) measuring protein S (vitronectin)-bound C terminal complex (SC5b-9). The assay has been used in clinical studies to quantify complement activation with high sensitivity and specificity (20). Liposome dispersions containing 60 mM total lipid were diluted 5-fold in human serum and were incubated for 30 min at 37 °C with shaking at 80 rpm. After incubation the samples were diluted 20-fold in the "sample diluent" of the kit and 100 µl aliquots from this mixture were applied into the wells of the ELISA plate, usually in duplicate. The assay was validated by repetitive measurement of randomly selected samples at different times with >1 year separation, using different batches of the SC5b-9 kit, and blinding the assayer(s) with regard to the identity of samples. In agreement with the manufacturer's specification of the SC5b-9 kit, these experiments revealed approximately 10-20% variation of SC5b-9 readings (16).

All liposome formulations were tested in serum samples of 4 to 5 different subjects. For each subject the % increase of SC5b-9 concentration was calculated and compared to the person's own baseline, referred to as "PBS control". Complement activation is presented as the mean % increase observed in the 4 – 5 subjects in which the formulations were tested.

In vivo porcine model of complement-related pseudoallergy

Experiments were performed in accordance with guidelines of the Committee on Animal Care of the Uniformed Services University of the Health Sciences. Castrated male Yorkshire swine (48-215 LB) were sedated with intramuscular ketamine, anesthetized with isoflurane and instrumented as described previously (13). In brief, a catheter was advanced via the right internal jugular vein into the pulmonary artery to measure pulmonary artery pressure (PAP), central venous pressure (CVP) and cardiac output (CO); another catheter was advanced through the left femoral artery into the proximal aorta to measure systemic arterial pressure (SAP). Systemic vascular resistance (SVR), left ventricular end-diastolic pressure (LEVDP) and pulmonary vascular resistance (PVR) were calculated from SAP, PAP, CO, and CVP by standard formulas. Cerebral blood flow, pCO_2 and ECG were recorded continually.

Liposomes were diluted in 1 ml PBS and injected into the pulmonary artery of pigs, via the pulmonary arterial catheter. Liposomes were flushed into the circulation with 10 ml PBS. Based on our previous finding that the hemodynamic effects of small liposome boluses were non-tachyphylactic and quantitatively reproducible in the same animal, we injected increasing amounts of the same type of liposomes in each pig until a reaction developed, or, in the absence of reaction, until a certain predetermined top dose was tested. The doses applied were in the 0.15-1.5 μmol lipid/kg (pig) range except for Doxil®, which, because of the severity of the reactions, had to be given at an almost 10-fold lower dose. The responses were graded as follows: none, no significant alteration in ECG or any hemodynamic parameters; mild, transient (< 2 min) <50 % changes in at least one of the following parameters: heart rate, ECG, SAP, PAP, pCO2; severe, up to 10 min and >50% changes in at least one of the above parameters plus bradyarrhythmia; lethal, circulatory collapse within 2 min requiring epinephrine and cardiac massage for resuscitation. Typically mean SAP falls from 110 to <40 mm Hg, mean PAP rises from 18 to a maximum (60 mm Hg), pCO_2 in expired gas drops from 32 to <20 mm Hg, tachycardia is followed by severe bradycardia with arrhythmia, leading to cardiac arrest and death.

Pharmacokinetics and therapeutic activity non-PEG-liposomes vs. PEG-liposomes

For comparative assessment of the pharmacokinetics of PEG-liposomes versus non-PEG liposomal formulations, DXP was encapsulated in liposomes composed of PEG-DSPE, DPPC and cholesterol (90 nm) and in two liposome types composed of DSPC and cholesterol (90 nm and 65 nm in size).

DXP can easily be detected in plasma and has the attractive property of being almost immediately and fully converted into dexamethasone when free in the circulation. As in a previous study liposomal glucocorticoid was shown to not leak encapsulated drug in the circulation, plasma levels of DXP could be regarded as a measure of liposome-associated drug (see Chapter 3). For assessment of DXP in plasma samples, plasma was extracted according to a method reported by Derendorf et al. and assayed with a reversed-phase HPLC method, using UV-absorption detection at 254 nm (21).

Rat experimental model of adjuvant arthritis

The Dutch Committee of Animal Experiments approved these animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (170-200 g) were obtained from Maastricht University, Maastricht, The Netherlands. Adjuvant arthritis was induced according to Koga and Pearson (22). Briefly, incomplete Freund's adjuvant containing heat-inactivated Mycobacterium tuberculosis was intracutaneously injected at the base of the tail. Paw inflammation started around day 10 after the immunization, reached maximal severity around day 20, after which the inflammation process gradually resolved. The rats were scored daily for the visual signs of inflammation. The severity of the joint inflammation was graded by assigning a score to each paw from 0 to 4, based on erythema, swelling and deformation of the joints. All rats were treated on day 15 post-induction with DXP in free form or DXP encapsulated in PEG-liposomes or DSPC-cholesterol liposomes (90 or 65 nm), when the average sum score of all paws of the rats in the experiment was around 7. The effect of treatment on clinical scores and body weight was monitored up to 4 weeks post-treatment.

Statistical Analysis

SC5b-9 values were expressed as mean ± SD of 4 – 5 subjects. One-sample t tests were used to determine whether the mean was significantly different from 100% (PBS value). Scores in the *in vivo* pig model were statistically evaluated with the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums). For evaluation of pharmacokinetics one-way analysis of variance was used, whereas for evaluation of the therapeutic activity also the Wilcoxon/Kruskal-Wallis test was applied. P values of less than 0.05 were considered significant.

RESULTS

Liposome characteristics

Table 1 shows the characteristics of the different liposome types used in this study. In general, the size of the liposomes ranged between 80 and 100 nm except for the DSPC-cholesterol liposomes of 65 nm. Polydispersity was always lower than 0.1 except in the case of Daunoxome®, which had a polydispersity of >0.2. The zeta-potential of PEG-liposomes was negative. Non-PEG-liposomes proved to be around neutral. Each liposome formulation contained around 40 µmol phospholipid/ml. The formulations prepared with DXP contained between 5 and 10 mg DXP/ml.

Table 1. Liposome composition and characteristics

Composition	Molar ratio	Mean diameter (nm)	Polydispersity index	Zeta potential (mV)
DPPC PEG-DSPE Chol	1.85 : 0.15 : 1.0	88	<0.1	-37
DSPC PEG-DSPE Chol	1.85 : 0.15 : 1.0	87	<0.1	-28
DSPC Chol	2.0 : 1.0	90	<0.1	-1
DSPC Chol	2.0 : 1.0	66	<0.1	-1
DSPC PEG-DSPE Chol + doxorubicin (Doxil®)	1.85 : 0.15 : 1.0	85	<0.1	-43
DSPC Chol + daunorubicin (Daunoxome®)	2.0 : 1.0	74	>0.2	-2

In vitro complement activation in human plasma

Fig 1 shows the % increase of the human serum SC5-b9 concentration after addition of liposomal formulations as compared to the baseline concentration. All liposome formulations induced a significant increase of SC5-b9 levels except for 65 nm DSPC-cholesterol liposomes.

In vivo porcine model of complement-related pseudoallergy

Table 2 shows that 5 out of the 6 formulations tested caused pseudoallergic reactions in the pigs. Liposomes without drug roughly induced reactions in half of the pigs that were tested. Both Doxil and Daunoxome caused severe to lethal reactions in the majority of the pigs in which they were tested. Especially Doxil proved to be a strong inductor of pseudoallergy, as the reactogenic dose range was an order of magnitude lower than that of other liposomes. The only formulation, which did not induce complement activation and pseudoallergy, was the DSPC-cholesterol liposome formulation of 65 nm.

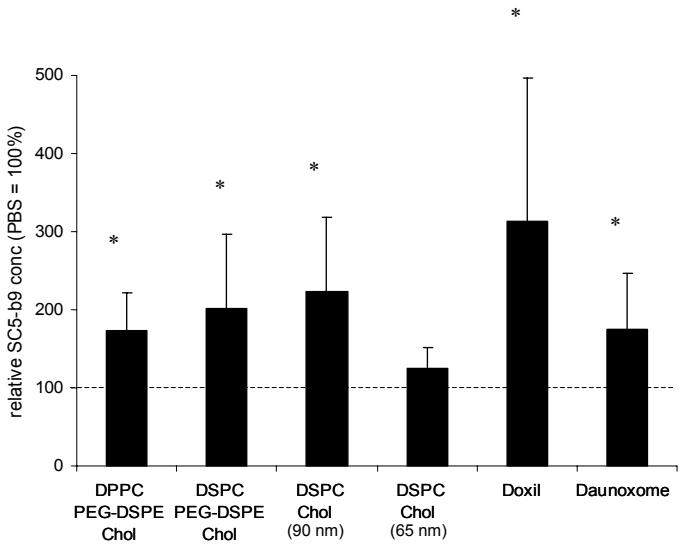

Figure 1. *In vitro* complement activation in human serum as measured by % increase of SC5-b9 levels as a result of addition of liposomes as compared to baseline SC5-b9 levels (addition of PBS). * = significant complement activation ($p < 0.05$, single sample t test). Data represent means of 4 – 5 subjects + SD.

Table 2. Hypersensitivity reactions in porcine model

liposomes	lipid dose (µmol/kg)	frequency of reactions	severity of reactions
DPPC PEG-DSPE Chol	0.17-1.39	4/6	None (2), Mild (1), Severe (1), Lethal (2)
DSPC PEG-DSPE Chol	0.16-1.97	2/4	None (2), Mild (0), Severe (1), Lethal (1)
DSPC Chol (90 nm)	0.16-1.85	5/11	None (6), Mild (3), Severe (2), Lethal (0)
DSPC Chol (65 nm)	0.16-1.54	0/8	None (8), Mild (0), Severe (0), Lethal (0)
Doxil®	0.02-0.27	12/14	None (2), Mild (3), Severe (8), Lethal (1)
Daunoxome®	0.18-0.73	7/8	None (1), Mild (2), Severe (1), Lethal (4)

Circulation kinetics: non-PEG-liposomes vs. PEG-liposomes

Fig 2 shows that non-PEG-liposomes composed of DSPC and cholesterol can behave as long-circulating as PEG-liposomes. DSPC-cholesterol liposomes of <70 nm had an even longer circulation half-life (appr. 36 hrs) as compared to PEG-liposomes (22 hrs).

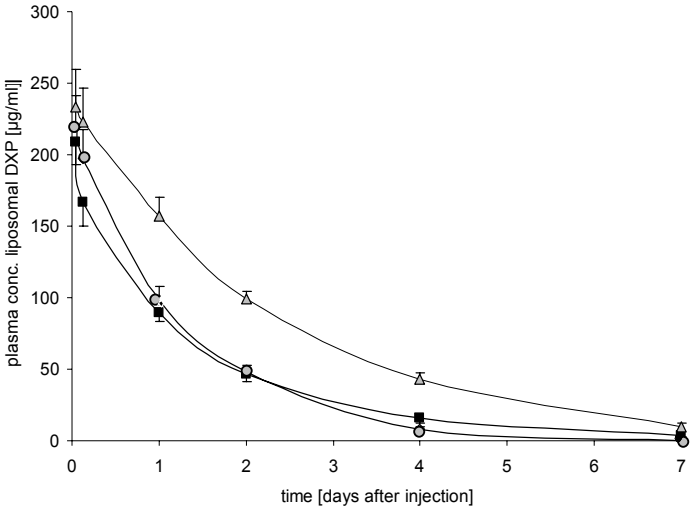

Figure 2. Plasma concentration-time curves of liposomal DXP after injection of 10 mg/kg DXP incorporated in DPPC-PEGDSPE-cholesterol liposomes (closed squares), DSPC-cholesterol liposomes of 90 nm (gray circles) and DSPC-cholesterol liposomes of <70 nm (gray triangles) in healthy rats. Data represent means +/- SD of groups of 4 rats.

Therapeutic activity of DXP-liposomes in rat adjuvant arthritis

Figure 3 A shows the effect of liposomal encapsulation of DXP on the therapeutic activity of the drug. Non-encapsulated DXP at 2 mg/kg reduced inflammation scores during one day, after which paw inflammation intensified again. In contrast, an equal dose of DXP encapsulated in PEG-liposomes led to complete remission of paw inflammation up to one week post-treatment. In Figure 3 B the effect of 1 mg/kg DXP in 90 nm and 65 nm DSPC-cholesterol liposomes without PEG is compared to an equal dose DXP-PEG-liposomes. Clearly, no difference in therapeutic activity is observed.

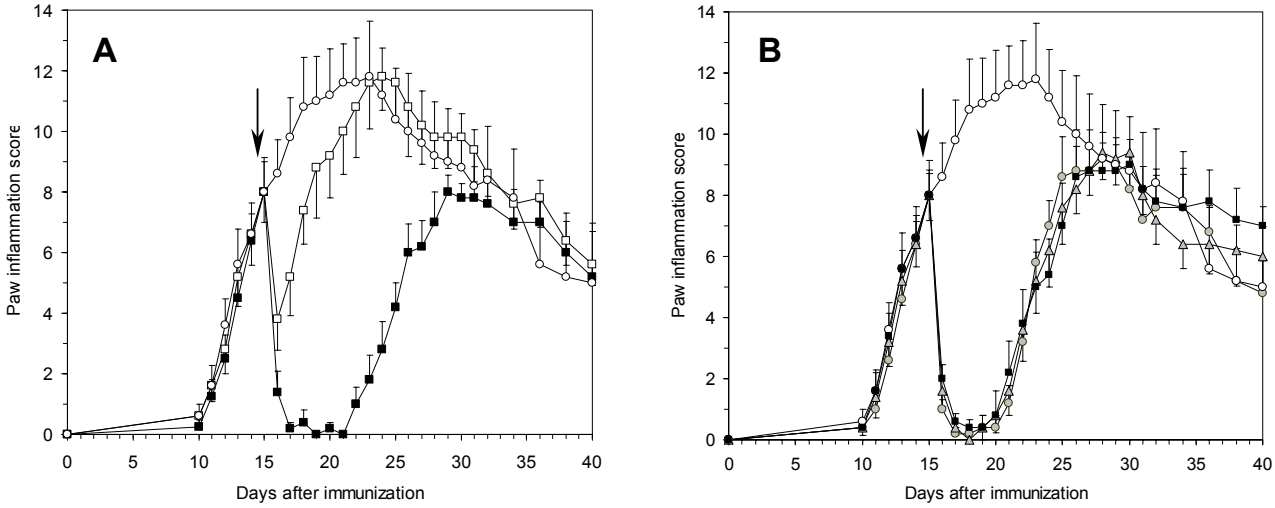

Figure 3. Therapeutic effect in rat adjuvant arthritis. (A) 2 mg/kg i.v. DXP in free form (open squares) given at day 15 (arrow), and 2 mg/kg DXP in PEG-liposomes (closed squares), both given as a single injection, and (B) 1 mg/kg DXP encapsulated in PEG-liposomes (closed squares), DSPC-cholesterol liposomes of 90 nm (gray circles) and DSPC-cholesterol liposomes of <70 nm (gray triangles) in adjuvant arthritis rats. Saline treated rats are used as controls (A and B) (open circles). Data represent means +/- SEM of groups of 5 rats.

DISCUSSION

Complement activation-related pseudoallergy in patients has been reported to occur with several lipid formulations (23). The most extensively studied liposome formulation in this respect is PEGylated liposomal doxorubicin (Doxil®, Caelyx®) (6-9). Of the formulations tested in the present study Doxil® indeed appeared to be the strongest inductor of complement activation. On the average, Doxil® caused a more than 3-fold increase of SC5-b9 in serum samples as compared to baseline values. Also, in the *in vivo* pig model, Doxil® caused reactions in 12 out of 14 pigs at 5- to 10-fold lower doses as compared to the other formulations tested.

This study confirms earlier observations that PEG-liposomes, also when doxorubicin is not encapsulated, can activate complement and cause hypersensitivity reactions in pigs (24). It is also in line with a report by Brouwers et al. on hypersensitivity reactions to ^{99}Tc-labeled empty PEG-liposomes in 3 out of 9 patients with Crohn's disease (10). Both the results of the *in vitro* SC5-b9 assay and the *in vivo* pig data reveal a response in roughly 50% of the test cases with PEG-liposomes. Remarkably, quite similar results were obtained with liposomes without PEG, indicating that PEG-PE and/or its negative charge cannot be the sole underlying cause of complement activation and subsequent hypersensitivity reactions.

In our studies, only the smallest size (mean diameter: 65 nm) DSPC-cholesterol liposome formulation was completely lacking complement-activating properties. Liposomes with the same composition but a somewhat larger mean diameter (90 nm) did show complement-activating activity, which indicates that size is an important factor in complement activation. Nevertheless, the presence of PEG-PE and/or its negative charge, and a relatively large size (i.e. ≥90 nm) may not represent the full list of risk factors for induction of complement activation. We found that Daunoxome®, a neutral, non-PEGylated DSPC-cholesterol formulation of daunorubicin, in our study showing a mean diameter of 74 nm, caused responses both *in vitro* and *in vivo* with a relatively high frequency. This observation is in line with the finding that Daunoxome® caused hypersensitivity reactions in 4 out of 15 of patients involved in a clinical phase II trial (25). Considering that Daunoxome® differed from the reaction-free empty control (DSPC-cholesterol) liposomes only in that it had substantially greater polydispersity index (see Table I), these observations suggest that the relatively large size distribution, probably caused by the presence of a fraction of larger liposomes or liposome aggregates among the small ones, is an additional major risk factor.

We have previously shown that Doxil® is a more effective complement activator than the corresponding empty PEGylated liposomes (24). Taken together with the present observations with Daunoxome®, it seems likely that the encapsulated doxorubicin and daunorubicin do play a role in C activation, despite the fact that both drugs are located within the liposome particles, apparently shielded from plasma. It has been observed that aggregates are present within these formulations (unpublished observations). Liposome aggregates, might be superior complement activators even in negligible quantities.

As small DSPC-cholesterol liposomes appeared to lack complement activating properties, it was of interest to evaluate whether these non-PEG-liposomes could also be used for passive drug targeting purposes. Therefore, we assessed the circulation time of DXP encapsulated in these liposomes in healthy rats. In addition, we evaluated the therapeutic activity of these liposomes in rat experimental arthritis. Our data showed that non-PEGylated DSPC-cholesterol liposomes circulated at least as long as PEG-liposomes. Interestingly, decreasing the size of DSPC-cholesterol liposomes from 90 nm to <70 nm extended the half-life to approximately 36 hrs in rats. In addition the therapeutic performance of these small non-PEGylated liposomes was similar to that of the PEG-liposomes.

In conclusion, the results presented in this study show that complement-related hypersensitivity reactions can occur either with or without the incorporation of PEG-PE in the liposomal formulation, indicating that PEG and/or a net negative surface charge may not be the (sole) key factor in the activation of complement by liposomes. Avoidance of complement activation may be achieved by sizing liposomes down to <70 nm with minimal polydispersity. The small, neutral DSPC-cholesterol liposome formulation, which in this study was completely lacking complement activation and hypersensitivity reactions, proved to be as valuable for targeting glucocorticoids to inflamed areas as 90 nm PEG-liposomes, which we used in most of the experimental work presented in this thesis. Therefore, the small non-PEGylated liposome formulation may be preferred over PEG-liposomes as carrier for passive drug targeting purposes.

ACKNOWLEDGEMENT

Dr. W. Jiskoot is gratefully acknowledged for critical reading of the manuscript.

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CHAPTER 9

A NOVEL FAMILY OF L-AMINO ACID-BASED BIODEGRADABLE POLYMER-LIPID CONJUGATES FOR THE DEVELOPMENT OF LONG-CIRCULATING LIPOSOMES WITH EFFECTIVE DRUG TARGETING CAPACITY

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ABSTRACT

Purpose. The objective of this study was to develop biodegradable polypeptide-lipid conjugates for the design of polymer-coated long-circulating liposomes (LCL).

Methods. Lipid conjugates of poly(hydroxyalkyl L-asparagine/L-glutamine) were synthesized and incorporated into 0.15 μm dipalmitoyl phosphatidylcholine (DPPC) - cholesterol liposomes. Circulation times and biodistribution were assessed in rats using a radioactive lipid marker. Evaluation of the therapeutic activity of prednisolone phosphate loaded in 0.1 μm PHEA-DPPC-cholesterol liposomes in a rat experimental arthritis model was performed to demonstrate the drug-targeting potential of the polymer-coated liposomes.

Results. Coating of liposomes with poly(hydroxyethyl L-asparagine) (PHEA) and poly(hydroxyethyl L-glutamine) (PHEG) extended the circulation half-life to a similar extent as poly(ethylene glycol) (PEG), which is normally used for the preparation of LCL. Glutamine polymers with a hydroxypropyl or a hydroxybutyl group instead of hydroxyethyl group also yield prolonged circulation, however, not to the same extent as PHEA/G. The pharmacokinetic properties of PHEA-liposomes were independent of the lipid dose even at very low lipid doses of around 50 nmol per rat. PLP was successfully entrapped in PHEA-liposomes. These liposomes were shown to be stable in the circulation and equally effective in rat experimental arthritis as PLP encapsulated in PEG-liposomes.

Conclusions. PHEA and PHEG are attractive alternative polymers for the design of LCL: their performance is similar to that of PEG-liposomes but they have the advantage of being biodegradable.

INTRODUCTION

Liposomes are small spherical particles that consist of one or more lipid bilayers enclosing an aqueous interior (1). Liposomes are highly suitable as drug carriers as they are composed of natural lipids and offer the opportunity to incorporate a wide variety of therapeutic agents for the purpose of drug-targeting (2). Biodistribution studies in laboratory animals showed that intravenously (i.v.) injected liposomes predominantly home to cells of the mononuclear phagocyte system (MPS) that is present in liver, spleen and bone marrow. As a result, liposomes show generally a short circulation half-life after i.v. administration (3,4).

The development of bilayer surface modifications that were able to reduce rapid uptake by the MPS, represented a major step forwards to the successful clinical application of liposomes (5). Incorporation of a lipid conjugate of the water-soluble polymer poly(ethylene glycol) (PEG) results in a polymeric layer around the liposome, which reduces the adhesion of plasma proteins that would otherwise cause rapid recognition of the liposomes by MPS-phagocytes. With a PEG-coating, liposomes oppose rapid uptake by the MPS and acquire a prolonged circulation property. With these so-called 'long-circulating liposomes' (LCL) drug targeting to tissues other than liver, spleen and bone marrow became possible. Indeed, LCL were shown to selectively accumulate at sites of enhanced vascular permeability as found in tumors and inflamed areas in the body (6,7). This form of targeted drug delivery resulted in increased therapeutic efficacy and/or reduced toxicity of several cytostatic, antifungal and antibacterial drugs and led to the market approval of liposomal doxorubicin as the first commercially available liposomal cytostatic agent (8-11).

Besides PEG a few other polymers have also been shown to induce prolonged circulation behavior of liposomes upon attachment to their surface (12). Woodle and coworkers investigated oxazoline-derived polymers, Maruyama et al. designed poly(glycerol)-coated liposomes, while Torchilin and coworkers developed several water-soluble vinyl-based polymers for the creation of long-circulating liposomes. All groups reported circulation half-lives comparable to PEG-liposomes (13-18). Up to now PEG-liposomes are the only polymer-coated liposomes that have been approved for clinical use. However, despite the fact that low-molecular weight PEG has been shown to be non-toxic and to be readily excreted by the kidneys, the biological fate of (liposome associated) PEG after cellular uptake is not known (19,20). Since PEG is expected not to be easily intracellularly degraded, it cannot be excluded that PEG may affect cell functioning at the long term (21).

It was the objective of this study to design a biodegradable and biocompatible polymer-lipid conjugate for the development of LCL. To achieve minimal toxicity of the polymer as well as the compounds that are formed upon biodegradation, we selected natural L-amino acids as starting material for the synthesis of polymer-lipid conjugates in this study. Besides the advantage of being intracellularly degradable, such poly(L-amino acid)-lipid conjugates are expected to be degraded by proteolytic enzymes that are present at pathological target tissues such as tumors and sites of inflammation. Therefore, these

polymer-lipid conjugates may allow the incorporation of bilayer functionalities that have to remain shielded in the circulation until arrival at the pathological target sites where they can perform their specific function.

The present study investigates the feasibility of polymers based on hydroxyalkyl derivatives of L-glutamine and L-asparagine as monomers. Poly(hydroxyethyl L-glutamine) (PHEG) was selected as a starting polymer as PHEG is known to be water-soluble, biocompatible and biodegradable by lysosomal peptidases from the papain family (22). Subsequently, structure-activity relationships were established by replacing the hydroxyethyl side group with other hydroxyalkyl groups and selecting L-asparagine as backbone monomer instead of glutamine. Different lipid molecules were evaluated to ensure stable anchoring of the polymer-lipid conjugates in the liposome bilayer. In addition, the effect of the polymer grafting density on the liposome surface and the effect of lipid dose on the circulation half-life were assessed. Finally, the feasibility of liposomal incorporation and targeting an anti-inflammatory glucocorticoid to inflamed sites in an experimental animal model of arthritis was evaluated.

MATERIALS AND METHODS

Synthesis and characterization of different PEG-lipid conjugates

PEG5000-lipid conjugates were synthesized from methoxy-PEG5000-isocyanate, commercially available from Shearwater Polymers, Huntsville Al, USA), and long-chain alkylamines: octadecyl amine (stearylamine) NH_2-$C_{18}H_{37}$ (ODA), having a single C_{18}-tail, dioctadecyl amine (distearylamine) NH-$(C_{17}H_{35})_2$ (DODA), and 1-heptadecyl-octadecylamine NH_2-CH-$(C_{17}H_{35})_2$ (HOA) both with a double alkyl tail. Isocyanate groups reacted with these primary and secondary alkyl amines rapidly at room temperature to form ureas of a general formula: mPEG-NH-CO-NH-R in case of a primary amine (ODA, R=$C_{18}H_{37}$ or HOA, R=CH$(C_{17}H_{35})_2$) or mPEG-NH-CO-N-R2 in case of a secondary amine (DODA , R being a C_{18}-tail).

All three PEG5000 derivatives were synthesized in exactly the same way. For the synthesis of the PEG5000-stearyl derivative a solution of 300 mg methoxyPEG-isocyanate (0.06 mmol, MW 5000, Shearwater Polymers) and 18 mg (0.07 mmol) ODA in 1.5 ml chloroform was stirred at room temperature for ca. 0.5-1 hour. The solution was then precipitated into 20-30 ml petroleum-ether. A fine white powder (220 mg, 75 %) was obtained after filtration and drying.

Characterization:
^{1}H-NMR ($CDCl_3$, δ in ppm relative to TMS):
PEG: 3.6 (CH_2-O)
Stearyl anchor: 1.2 (CH_2) & 0.8 (CH_3)
The ratio of PEG and stearyl peak integrals confirms the presence of one stearyl group per PEG moiety.

Synthesis and characterization of PHEA-DODASuc

Synthesis of poly(L-hydroxyethyl asparagine)-N-succinyl-dioctadecylamine (PHEA-DODASuc) is schematically represented in Figure 1 A. To a solution of 3 g β-benzyl L-aspartate N-carboxy anhydride (NCA) (synthesized as described by Fuller et al. (23)) dissolved in 9 ml dry dimethylformamide (DMF) (Aldrich-Chemie, Steinheim, Germany) was added 0.3 ml of a 2 M solution of methylamine in tetrahydrofurane (THF) (Aldrich) as initiator. The solution (initially clear, after ca. 2 hrs cloudy) was stirred for one day under a nitrogen atmosphere at room temperature and then precipitated into water (150 ml), collected by filtration and dried. Yield: 2 g poly(benzyl-L-aspartate) (PBLA).

Polymer-lipid coupling was performed to obtain the PBLA-DODASuc-conjugate. Briefly, a solution of 2 g PBLA in 5 ml chloroform containing ca. 150 ml triethylamine (Merck, Darmstadt, Germany) was added to a solution of 200 mg dicyclohexylcarbodiimide DCC (Acros Chimica, Geel, Belgium), 15 mg 4-(dimethylamino) pyridinium-4-toluene sulfonate (DPTS) (Acros) and 350 mg N-succinyl-dioctadecylamine (DODASuc; for synthesis

see (24)) in 6 ml chloroform, that had been stirred for 1 hour. The mixture was stirred for one day and then precipitated into methanol. The polymeric product was filtered off and dried *in vacuo*. Yield: 1.4 g PBLA-DODASuc. Aminolysis of 1.4 g PBLA-DODASuc was performed with ca. 4 ml ethanolamine (Aldrich), using 0.4 g 2-hydroxypyridine (Aldrich) as a catalyst, in 10 ml DMF solution at 40 °C for 1 day yielding PHEA-DODASuc. Yield: 0.8 g after dialysis (molecular weight cut off 500) and freeze-drying.

NMR (DMSO-d6) (δ in ppm relative to TMS):
distearyl: 0.8 (CH_3), 1.2 (CH_2), 1.4 (CH_2-N)
PHEA: 2.4-2.8 (β-CH_2), 3.2 & 3.4 (hydroxyethyl), 4.6 (α-CH + OH), 7.8-8.5 (NH)
From the ratio of integrals of the distearyl signals and the α-CH signal the molecular weight of the polymer part of the conjugate was calculated to be ca. 3000 g/mol.

Synthesis and characterization of poly(L-hydroxyalkyl glutamine)-DODASuc-conjugates

Synthesis of poly(hydroxyethyl glutamine) (PHEG)-lipid conjugates is schematically shown in Figure 1 B. To a solution of 3 g γ-benzyl-L-glutamate NCA (for synthesis see (23)) in 8 ml dry DMF was added a solution of 0.1 g N-BOC-1,4-diaminobutane (Fluka, Zwijndrecht, The Netherlands) in chloroform as initiator. This solution was stirred for 1 day under a nitrogen atmosphere at room temperature. After precipitation into ca. 100 ml methanol the polymer was filtered off and dried, yielding 2 g PBLG with a BOC-protected amino end group. To remove BOC, a solution of 1.7 g PBLG-diaminobutane-BOC in 12 ml 2 M HCl/dioxane was stirred for 4 hrs and then added dropwise to ca. 150 ml water in which appr. 10 g $NaHCO_3$ was dissolved. The product was filtered off, washed with water and dried *in vacuo*. Yield: 1.4 g PBLG-diaminobutane. Deprotection was complete as demonstrated by NMR analysis.

To obtain the PBLG-DODASuc-conjugate, 340 mg DODASuc, 180 mg DCC and 15 mg DPTS were dissolved in 5 ml chloroform. The solution was stirred for 1 hour at room temperature. Next, a solution of 1.4 g PBLG-diaminobutane and 120 mg triethylamine in 8 ml chloroform was added. After stirring overnight at room temperature the obtained solution was added dropwise to an excess of methanol (ca. 150 ml). The polymeric product was filtered off, washed and dried. Yield: 1.2 g PBLG-DODASuc.

^{1}H-NMR ($CDCl_3$) (δ in ppm relative to TMS):
distearyl signals at 0.8-0.9 (CH_3) and 1.2-1.4 (methylene protons)
PBLG: 2.2 & 2.6 (β,γ-CH_2), 4.0 (α-CH), 5.0 (benzyl CH_2), 7.3 (phenyl)

Aminolysis with ethanolamine was performed to obtain PHEG-DODASuc: 1.2 g PBLG-DODASuc (see above) and 0.5 g 2-hydroxypyridine were dissolved in 10 ml DMF. Then ca. 4 ml ethanolamine was added dropwise. After stirring for 24 hrs at 40 °C under a nitrogen atmosphere the solution was precipitated into ca. 200 ml diethylether. The precipitate was dissolved in water, dialyzed (molecular weight cut off 500) and subsequently freeze-dried yielding 0.8 g PHEG-DODASuc conjugate.

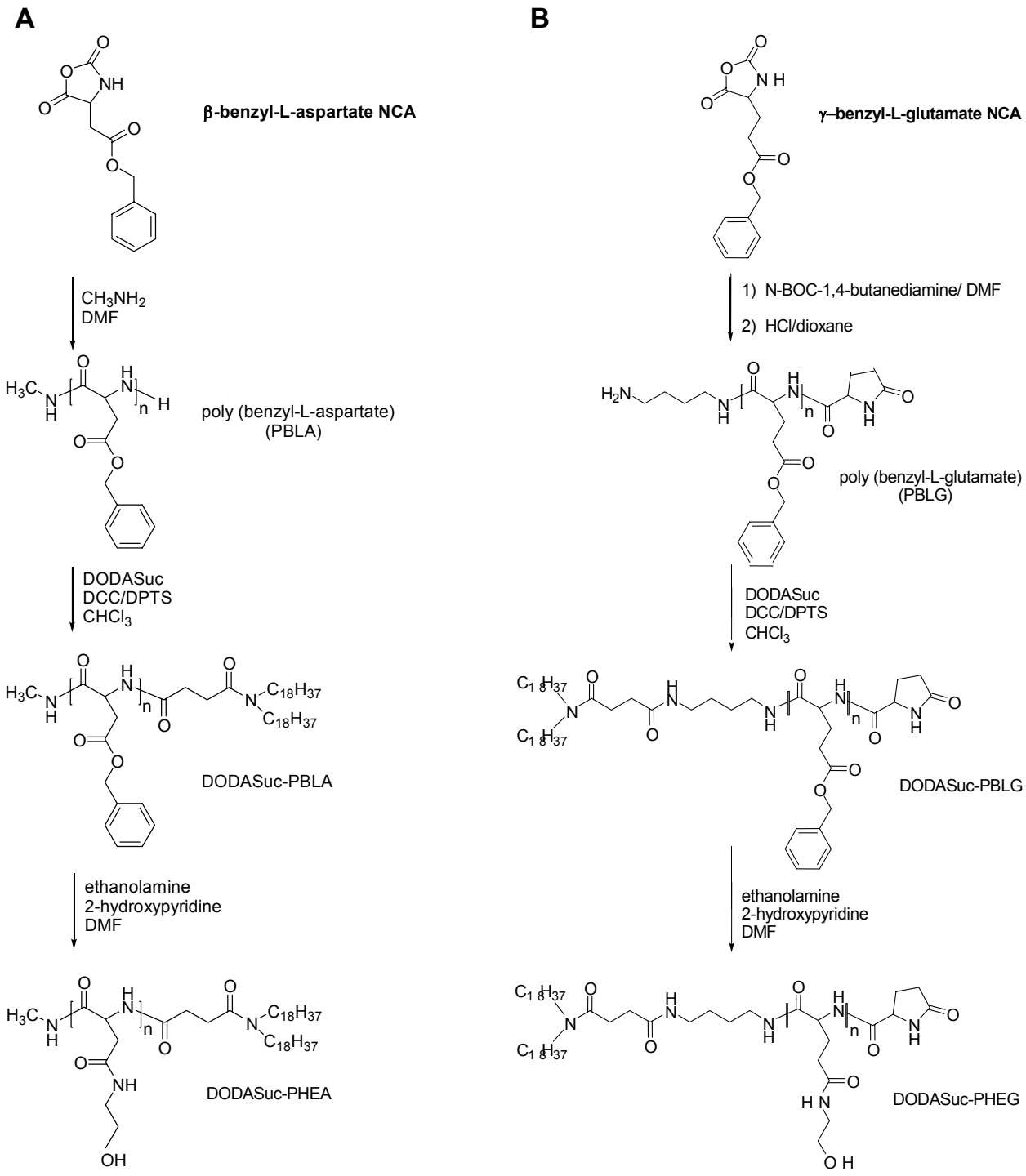

Figure 1. Synthesis of poly(hydroxylalkyl L- asparagines/glutamine)-lipid conjugates. (A) Schematic representation of PHEA-DODASuc synthesis strategy. (B) Schematic representation of the synthesis of PHEG-DODASuc. Note that DODASuc is attached to PHEA at the amino end group (on the right) of the poly(amino acid) back bone, whereas to PHEG it is attached to the carboxylic end (on the left, using diaminobutane to connect the poly(amino acid) back bone to DODASuc).

1H-NMR (DMSO-d6) (δ relative to TMS):
distearyl signals at 0.8-0.85 (CH_3) and 1.2-1.5 (methylene protons)
PHEG: 1.7-2.2 (β,γ-CH_2), 3.1 & 3.3 (hydroxyethyl), 4.2 (α-CH), 4.7 (OH), 7.8 & 8.2 (NH)
From the ratio of integrals of the distearyl signals and the α-CH signal molecular weight of PHEG was calculated to be ca. 4000.
By varying the monomer/initiator ratio similar conjugates with different PHEG molecular weights (3000 and 8000) were synthesized.

Maldi-TOF confirms the molecular structure of the PHEG-DODASuc conjugate as depicted in Figure 1 A.
Na+-adduct: m/z 3064.5 (n=13), 3236.1 (n=14), 3408.7 (n=15), 3580.6 (n=16), 3752.9 (n=17), 3924.7 (n=18), 4096.7 (n=19), 4268.4 (n=20), 4441.1 (n=21), 4613.3 (n=22), 4785.1 (n=23), etc.

The same procedure of aminolysis but with 3-propanol amine instead of ethanolamine yielded PHPG-DODASuc:

NMR (DMSO-d6) (δ in ppm relative to TMS):
distearyl signals at 0.8-0.85 (CH_3) and 1.2-1.5 (methylene protons)
PHPG: 1.7-2.2 (β,γ-CH_2), 1.5 & 3.1 & 3.3 (hydroxypropyl), 4.2 (α-CH), 4.6 (OH), 7.8 & 8.2 (NH)

Maldi-TOF:
Na+-adduct: m/z 3623 (n=15), 3810 (n=16), 3996 (n=17), 4182 (n=18), 4368 (n=19), 4555 (n=20), etc.

To obtain PHBG-DODASuc, the same procedure of aminolysis of PBLG-DODASuc was repeated with 4-butanolamine (Merck). Stirring was performed for 48 hrs instead of 24 hrs at 40 °C.

NMR (DMSO-d6) (δ in ppm relative to TMS):
distearyl signals at 0.8-0.85 (CH_3) and 1.2-1.5 (methylene protons)
PHBG: 1.7-2.2 (β,γ-CH_2), 1.4 & 3.1 & 3.3 (hydroxybutyl), 4.2 (α-CH), 4.5 (OH), 7.8 & 8.2 (NH)

Preparation of radiolabeled liposomes for comparative pharmacokinetics

Liposomes were prepared as described previously (25). Briefly, a lipid mixture in ethanol with a molar ratio composition of 1.85:0.15:1.0 (DPPC:polymer-lipid conjugate:cholesterol) was prepared. Such molar ratio results in liposomes containing 7.5% polymer-lipid conjugate as a percentage of the total amount of phospholipid. To the mixture [^{3}H]-cholesteryl oleylether was added as a non-degradable liposome lipid phase marker. A lipid film was created by rotary evaporation under reduced pressure. The lipid film was hydrated with phosphate buffered saline (PBS) at an initial total lipid concentration of 20 μmol/ml.

The liposomes were sized by multiple extrusion using a medium pressure extruder equipped with two stacked polycarbonate membrane filters, one with a pore size of 200 nm on top of one with 100 nm pores. Components that were not incorporated in liposomes were removed by gel filtration on a PD-10 column (Pharmacia, Uppsala, Sweden) eluted with PBS.

Characterization of liposome preparations

Radioactivity of the liposomal dispersions was assayed in an Ultima Gold liquid scintillation cocktail purchased from Hewlet Packard (Groningen, The Netherlands) and counted in a Philips PW 4700 liquid scintillation counter. Lipid content of the liposomal dispersion was determined by assessing the radioactivity of the liposomes before and after preparation. The mean particle size of the liposomes was determined by dynamic light scattering with a Malvern 4700 system (Malvern, UK). The mean size ranged between 140 and 160 nm. In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1; 0 indicating that a complete monodisperse system is obtained, whereas 1 indicates maximal variation in particle size). All liposome preparations used had a polydispersity index of below 0.15. In Table I the composition and characteristics of the different liposome types are summarized. Liposome preparations were stored under nitrogen at 4 °C and used within one week after preparation.

Table I. Liposome composition and characteristics. Means of 3 measurements are shown.

	Grafting density (%)	Composition DPPC:Chol:PLC*	Mean diameter (nm)	Polydispersity index
BARE(no polymer)	0	2:1	151 ± 2	0.13 ± 0.02
PEG-DSPE	7.5	1.85:1.0:0.15	139 ± 2	0.11 ± 0.01
PHEG-DODASuc	1	1.98:1.0:0.02	151 ± 2	0.09 ± 0.01
	2.5	1.95:1.0:0.05	156 ± 2	0.08 ± 0.01
	7.5	1.85:1.0:0.15	151 ± 2	0.07 ± 0.02
	15	1.85:1.0:0.30	160 ± 1	0.07 ± 0.01
PHEA-DODASuc	7.5	1.85:1.0:0.15	146 ± 2	0.09 ± 0.02
PHPG-DODASuc	7.5	1.85:1.0:0.15	153 ± 1	0.09 ± 0.02
PHBG-DODASuc	7.5	1.85:1.0:0.15	148 ± 2	0.07 ± 0.04

* PLC: Polymer-lipid conjugate

Comparative pharmacokinetics of polymer-coated ^{3}H-labelled liposomes in rats

Male Wistar rats with an approximate body weight of 200 g were used (outbred, SPF-quality, Utrecht University, The Netherlands). Besides the different poly(hydroxyalkyl L-amino acid)-coated liposomes, non-polymer-coated ('bare') liposomes and liposomes coated with PEG2000 coupled to distearoyl phosphatidylethanolamine (PEG-DSPE) were prepared and evaluated as 'negative' and 'positive' controls respectively. Single-dose intravenous injections of liposomal preparations containing 5 μmol total lipid and approximately 50 kBq of radioactivity, were given in the tail vein. Blood samples of 100 μg were collected from the opposite tail vein of each rat at the following time points post-injection: 5 minutes and 1, 4, 8, 24 and 48 hours. Radioactivity in blood samples was determined by adding Solvable tissue solubilizer (NEN, Dreieich, Germany) and 35% hydrogen peroxide. After overnight incubation the samples were assayed in Ultima Gold scintillation cocktail (Packard BioScience B.V., Groningen, The Netherlands) and counted for radioactivity with a Philips PW 4700 liquid scintillation counter. At 48 hrs post-injection liver and spleen were dissected, homogenized and processed according to the same method as described for the blood samples. Besides tissue and blood samples also the radioactivity of the injected dose was counted. The % injected dose in the organs was calculated by dividing the total radioactivity of the organs by the injected dose. To obtain the %injected dose values in the circulation, the radioactivity of the blood samples was multiplied with a factor 10 times the total mass of the blood in grams (calculated as 7% of the total body weight) and divided by the injected dose. The results are presented as the mean ± standard deviation of the percentage of the injected dose of 4 rats.

Liposome preparation for assessing drug targeting potential

Liposomes containing the anti-inflammatory glucocorticoid prednisolone phosphate (PLP) were prepared as described previously (see Chapter 3). In brief, a lipid film containing 7.5% PHEA-DODASuc or PEG-DSPE was created by rotary evaporation and hydrated with a solution of 100 mg/ml prednisolone phosphate (PLP) (Bufa, Uitgeest, The Netherlands) in water at a initial lipid concentration of 100 μmol lipid per ml dispersion. Liposomes were sized to approximately 90 nm by multiple extrusion. A smaller size than the liposomes used in the comparative pharmacokinetic studies was chosen, as this may further increase target localization of the liposomes. PLP and was removed by repeated dialysis using Slide-A-Lyzer dialysis cassettes with a molecular weight cut off of 10.000 (Pierce, UK) against PBS. Phospholipid content was determined with a phosphate assay in the organic phase after extraction of the liposomal preparations with chloroform (26). The aqueous phase after extraction was used for determining the PLP content by HPLC. Each ml liposomal preparation contained around 5 mg PLP and approximately 60 μmol phospholipid.

Pharmacokinetics of PLP encapsulated in polymer-coated liposomes

For assessment of *in vivo* stability in the circulation and pharmacokinetic behavior of PLP in polymer-coated liposomes, the PLP plasma concentration was measured at the time of injection, at 24 and 48 hrs post-injection. The PLP-plasma concentration was compared with PLP in PEG-liposomes, as the latter has been shown to be completely stable in the circulation by comparing the plasma concentration of a radioactive liposome marker to the plasma concentration of encapsulated PLP (see Chapter 3). In the same study plasma levels of PLP could hardly be detected after i.v. injection of free PLP, which strongly suggests that all PLP measured in plasma after injection of liposomal PLP must be liposome-associated. To measure PLP in plasma samples, plasma was extracted according to a method reported by Derendorf et al. (27). The extracts were assayed with a reversed-phase HPLC method, using UV-absorption detection at 254 nm.

Therapeutic activity of PLP-containing polymer-coated liposomes in adjuvant arthritis

The Dutch Committee of Animal Experiments approved the animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (170-200 g) were obtained from Maastricht University, Maastricht, The Netherlands. Adjuvant arthritis was induced according to Koga and Pearson (28). Briefly, incomplete Freund's adjuvant containing heat-inactivated Mycobacterium tuberculosis was subcutaneously injected at the base of the tail. Paw inflammation started around day 10 after the immunization, reached maximal severity around day 20, after which the inflammation process gradually resolved. The rats were daily scored for the visual signs of inflammation. All rats were treated on day 15 post-immunization, when the average score of all rats in the experiment is about half the maximal scores reached in these experiments. The effect of treatment on clinical scores and body weight was monitored up to 4 weeks post-treatment.

Statistical analysis

For statistically assessing and comparing therapeutic efficacy in different groups the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups regarding other parameters, one-way analysis of variance was used. P values of less than 0.05 were considered significant.

RESULTS

Synthesis of PHEA and PHEG-DODASuc-conjugates

Poly(hydroxyethyl L-asparagine) (PHEA) and poly(hydroxyethyl L-glutamine) (PHEG) were synthesized starting from benzyl L-aspartate NCA and benzyl L-glutamine NCA, respectively, as shown in Figure 1. Polymerization of these L-amino acid benzyl ester NCA monomers was followed by aminolysis with alkanolamines. Molecular weights in the range of 2000-5000 were obtained via a primary amine initiated polymerization of these side group-protected amino acid NCA monomers. The molecular weight of the polymer is controlled by the molar ratio of monomer/initiator. N-succinyl-dioctadecylamine (DODASuc) (lipid anchor) could be coupled to the polypeptide's amino end group. Polymerization of benzyl-L-aspartate initiated by a primary amine (e.g. methylamine) yields a polypeptide (PBLA) with an amino end group. In contrast, polymerization of benzyl-glutamate NCA initiated by a primary amine results in the formation of a polypeptide (PBLG) without an amino end group. As could be concluded from Maldi-TOF, a 5-membered lactam end group is formed by an intramolecular reaction of the amino end group with the benzyl ester group. The terminal amino group is lost.

Conjugation of DODASuc to PBLG, therefore, requires a slightly modified approach: first, benzyl-L-glutamate NCA is polymerized using BOC-butanediamine (one amino group is BOC (tertiary butoxycarbonyl)-protected; the other amino group is not) as an initiator. The resulting PBLG containing a protected amino group is deprotected and then coupled to DODASuc using dicyclohexyl carbodiimide (DCC). Amphiphilic conjugates resulted after the polypeptides were made water-soluble by the aminolysis reaction of the benzyl ester side groups with alkanolamines (e.g., ethanolamine).

Preparation and characterization of polymer-coated liposomes

In Table I the characteristics of the polymer-coated liposomes are presented. The resulting mean diameter and polydispersity of PHEG-, PHPG-, PHBG- and PHEA-coated liposomes are comparable to those of PEG-liposomes. All preparations appeared to be physically stable upon storage and no signs of aggregation were found.

Selection of suitable anchor molecules

Figure 2 A shows the plasma concentration-time profiles of liposomes coated with different PEG5000-conjugates after i.v. injection. For the evaluation of the anchor molecules PEG5000 was chosen instead of PEG2000 as a molecular mass of PEG5000 more closely resembles the molecular mass of most of the polymers tested in this study. We coupled PEG5000 to a series of lipid anchor molecules and compared liposomes coated with these conjugates to liposomes without PEG ('bare' liposomes). Clearly, octadecyl amine (ODA) as a lipid anchor results in little prolongation of circulation time, suggesting that one single C_{18}-tail is not sufficient for stable grafting. Dioctadecyl amine (DODA) and heptadecyl octadecyl amine

(HOA) yield improved prolongation of circulation behavior, similar to the phospholipid anchor distearyl phosphatidylethanolamine (DSPE), which is generally used for stable grafting of PEG on the liposome bilayer. Apparently, two alkyl tails are required for sufficient grafting stability. We selected DODA as the standard anchor molecule for synthesis of PHEA- and the different poly(hydroxyalkyl L-glutamine)-lipid conjugates.

Figure 2 B shows the tissue distribution to the MPS organs. The DODA- and HOA-PEG conjugates significantly reduce the hepatosplenic uptake of liposomes. As hepatosplenic uptake is the main cause of liposome elimination from the circulation, these data are in agreement with plasma concentration-time profiles shown in Figure 2 A.

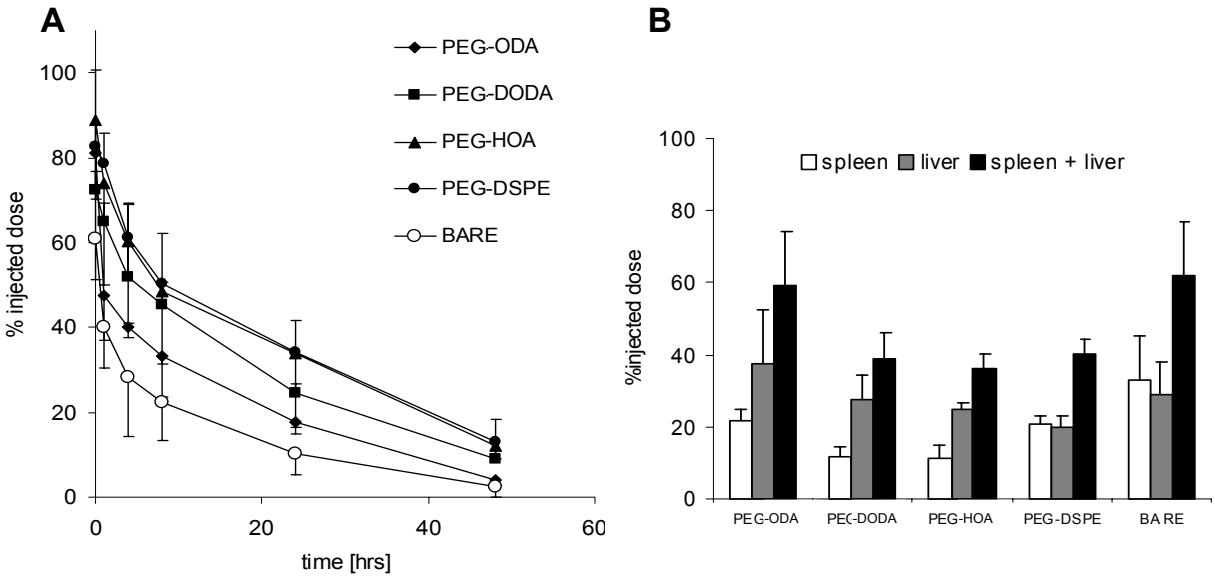

Figure 2. Pharmacokinetics and distribution to the MPS of PEG5000 conjugated with different anchor molecules incorporated with a grafting density of 7.5% in 150 nm DPPC-cholesterol liposomes. (A) %-injected dose in blood-curves of PEG5000–ODA (closed diamonds),PEG5000–DODA (closed squares), PEG5000–HOA (closed triangles), PEG5000–DSPE (closed circles), and bare liposomes without polymer-lipid conjugate (open circles). (B) Distribution to spleen (open bars), liver (gray bars) and total distribution to the MPS (liver and spleen) (black bars). Results are expressed as the mean percentage of the injected dose of 4 rats ± SD.

Successful prolongation of circulation half-life with PHEG- and PHEA-DODASuc

In Figure 3 A it is shown that coating liposomes with 7.5% PHEG4000 or PHEA3000 and PHEA5000 all coupled to N-succinyl-DODA (DODASuc) results in almost similar circulation behavior as compared to 7.5% PEG2000-DSPE, the conjugate which is most often used for the preparation of LCL. These results are in line with the tissue distribution data shown in Figure 3 B, which shows that all four polymer-lipid conjugates significantly reduce MPS uptake from the circulation.

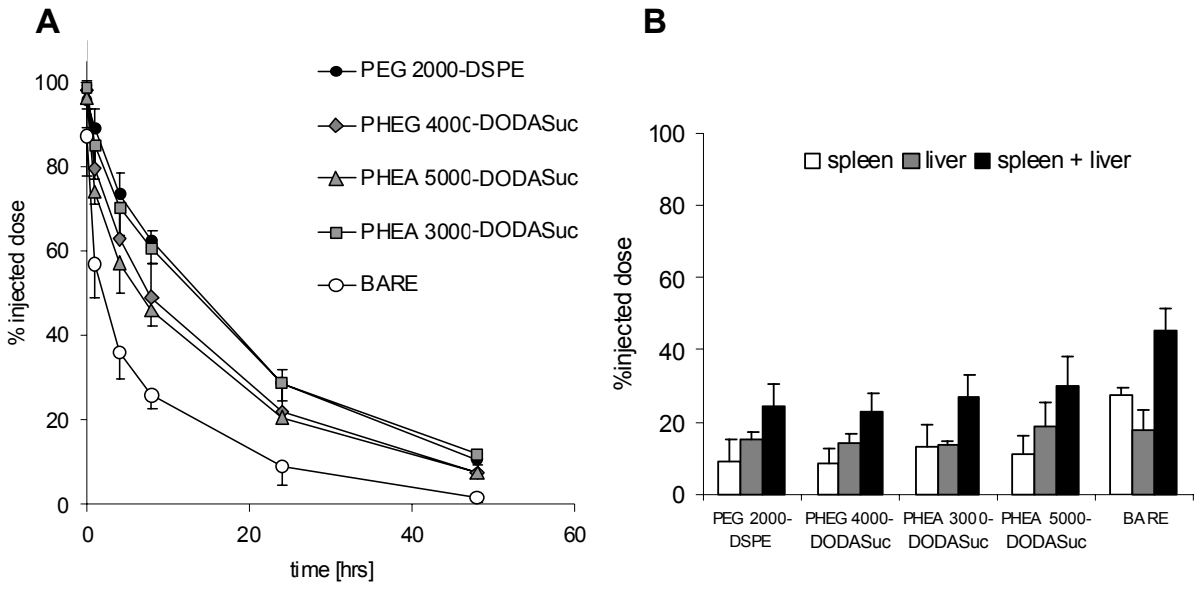

Figure 3. Pharmacokinetics and distribution to the MPS of PHEA and PHEG versus PEG incorporated with a grafting density of 7.5% in 150 nm DPPC-cholesterol liposomes. (A) %-injected dose in blood-curves of PEG2000–DSPE (closed circles), PHEG4000–DODASuc (gray diamonds), PHEA5000–DODASuc (gray triangles), PHEA3000–DODASuc (gray squares, and bare liposomes without polymer-lipid conjugate (open circles). (B) Distribution to spleen (open bars), liver (gray bars) and total distribution to the MPS (liver and spleen) (black bars). Results are expressed as the mean percentage of the injected dose of 4 rats ± SD.

Selection of optimal grafting density with PHEG-DODASuc

In Table II the effect of different grafting densities of PHEG4000 on circulation behavior and hepatosplenic uptake is shown. Although plasma concentration-time profiles show little differences at 4 and 24 hrs, hepatosplenic uptake at 48 hrs indicates that decreasing the grafting density to 2.5% and 1% or increasing the density to 15% leads to enhanced removal of liposomes by the MPS.

Table II. Percentage of injected dose of PHEG-liposomes in the circulation at 4 and 24 hrs post-injection and uptake by MPS organs at 48 hrs. Mean ± SD of 4 rats per group

PHEG Grafting density	Blood circulation		Liver uptake	Spleen uptake
	4 h	24 h	48 h	48 h
1%	55 ± 2	22 ± 1	52 ± 9	11 ± 6
2.5%	56 ± 8	22 ± 1	38 ± 7	15 ± 2
7.5%	61 ± 6	23 ± 4	23 ± 5	7 ± 2
15%	49 ± 4	17 ± 3	32 ± 5	9 ± 1

Effect of the side group

Introduction of longer hydroxyalkyl side groups to the poly(L-glutamine) back bone, such as hydroxypropyl and hydroxybutyl instead of hydroxyethyl, results in reduced prolongation of circulation time and enhanced MPS uptake to the level of 'bare' liposomes (Figure 4). The hydroxyethyl side group appears to be the optimal side group for the poly(hydroxyalkyl L-glutamine)-lipid conjugates.

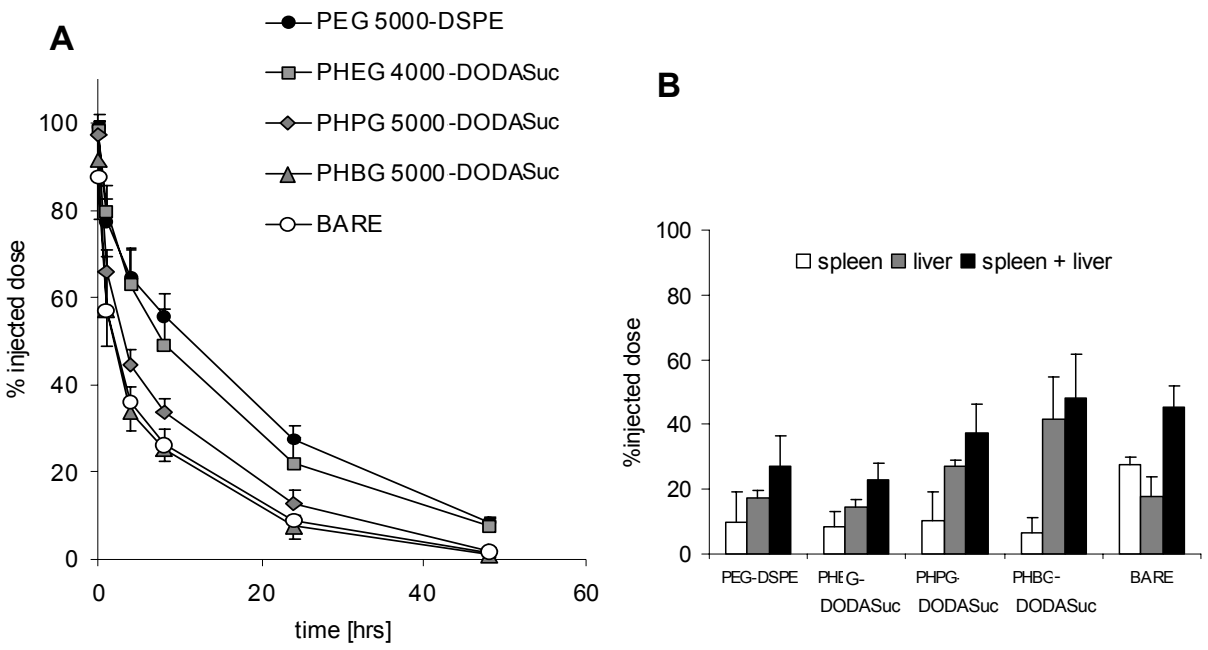

Figure 4. Pharmacokinetics and distribution to the MPS of different poly(hydroxyalkyl L-glutamine)s incorporated with a grafting density of 7.5% in 150 nm DPPC-cholesterol liposomes. (A) %-injected dose in blood-curves of PEG2000–DSPE (closed circles), PHEG4000–DODASuc (gray squares), PHPG5000–DODASuc (gray diamonds), PHBG5000–DODASuc (gray triangles), and bare liposomes without polymer-lipid conjugate (open circles). (B) Distribution to spleen (open bars), liver (gray bars) and total distribution to the MPS (liver and spleen) (black bars). Results are expressed as the mean percentage of the injected dose of 4 rats ± SD.

Table III. Percentage of the injected dose of liposomes in circulation at 4 and 24 hrs post-injection. Mean ± SD of 4 rats per group.

	PEG-liposomes		PHEA-liposomes	
Lipid dose	4 h	24 h	4 h	24 h
5 µmol	66 ± 7	29 ± 6	57 ± 4	27 ± 9
0.5 µmol	71 ± 10	26 ± 5	57 ± 5	18 ± 2
0.05 µmol	46 ± 7	12 ± 3	61 ± 4	20 ± 1
0.005 µmol	5 ± 3	1 ± 1	33 ± 5	9 ± 2

Effect of lipid dose

Table III shows the % injected dose of liposomes still present in the circulation at 4 and 24 hrs post-injection of PEG-coated liposomes and PHEA-coated liposomes, both at 4 different dose levels ranging from 0.005 to 5 μmol lipid per rat. At a dose of 0.05 μmol and especially at 0.005 μmol PHEA-liposomes show improved circulation behavior as compared to PEG-liposomes. PEG-liposomes are very rapidly eliminated at the 5 nmol dose level, they are hardly present in the circulation at 24 hrs post-injection, whereas still 9% of the injected PHEA-liposomes circulates at 24 hrs at the same dose level.

Drug targeting potential

Prednisolone phosphate (PLP) was encapsulated in both PEG- and PHEA-liposomes and injected in adjuvant arthritis rats. In this experimental arthritis model we previously showed that the therapeutic activity of PLP was dramatically increased by encapsulation in PEG-liposomes. Figure 5 A shows the plasma concentration of liposomally encapsulated PLP. The % fractions of the injected dose of PLP in PHEA-liposomes in the circulation at 24 and 48 hrs equals PLP in PEG-liposomes. As PLP-PEG-liposomes composed of DPPC and cholesterol have been shown not to leak PLP in the circulation, such may therefore also be assumed for PLP-PHEA-liposomes. The circulation half-life of these liposomes is somewhat longer than reported with the radiolabeled liposomes in this study. However, this is most likely a result of a relatively small diameter of approximately 90 nm as compared to the radiolabeled liposomes (between 140 and 160 nm). Figure 5 B shows that 10 mg/kg PLP-PHEA-liposomes induces a similar reversal of the inflammation reaction as 10 mg/kg PLP-PEG-liposomes. Both preparations induce a strong anti-inflammatory effect lasting for two weeks.

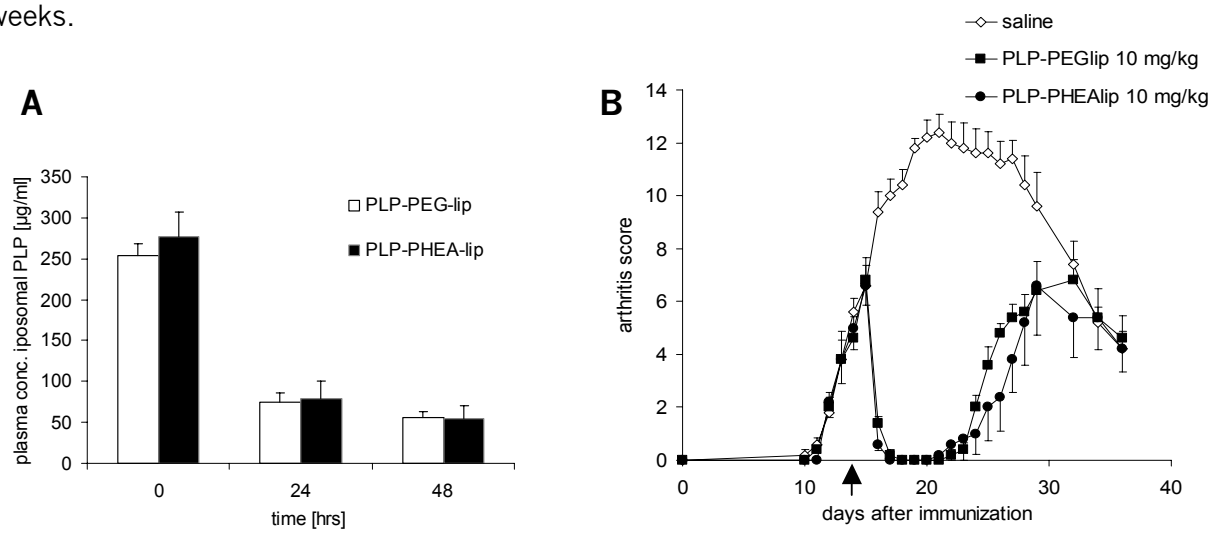

Figure 5. Pharmacokinetics and therapeutic activity of PHEA- versus PEG-liposomal PLP in rat experimental arthritis. A) Plasma concentration at 0, 24, and 48 hrs post-injection of 10 mg/kg PLP in PHEA-liposomes (open bars) and PEG-liposomes (closed bars). Results are expressed as the mean concentration (μg/ml) of 5 rats + SD. B) Daily arthritis score after induction of adjuvant arthritis at day 0 and treatment at day 15 (arrow) with 10 mg/kg PLP (120 μmol/kg phospholipid) in PHEA-liposomes (closed circles), 10 mg/kg in PEG-liposomes (closed squares), and saline (open diamonds). Results are expressed as the mean of 5 rats ± SEM.

DISCUSSION

In this paper biodegradable polymer-lipid conjugates based on amino acids are evaluated regarding their capacity to confer a long-circulation property to liposomes upon attachment to their lipid bilayer surface. Besides the polymer itself, also the choice of the lipid bilayer anchor deserves attention, as stable grafting of a polymer to the liposome bilayer is essential for achieving long-circulating behavior. Therefore, in this study the suitability of different lipid anchor molecules was evaluated with PEG5000 as a model polymer (Figure 2). The results show that successful prolongation of liposome circulation time can only be realized with anchor molecules with two stearyl chains. PEG coupled to DODA and HOA resulted in similar prolongation of circulation behavior as PEG-DSPE, which is at present most often used in liposome technology. In agreement with the findings of Webb et al., coupling of the polymer to a single alkyl chain yielded inferior results, most likely due to insufficient grafting stability (29). As DODA was easier to obtain from commercial sources than HOA, all polypeptides were tested in subsequent experiments with DODA as liposome anchor molecule.

The approach of L-amino acid-based polymer-lipid conjugates proved to be successful with PHEG4000, PHEA3000 and -5000, all coupled to DODASuc. The plasma concentration-time profiles as well as uptake by liver and spleen showed that these polymer-lipid conjugates prolonged the circulation time and reduced the uptake by the MPS to the same extent as PEG2000-DSPE (Figure 3). As has been shown in literature for PEG, lowering the grafting density of PHEG to 1 mol % resulted in increased uptake by the MPS (30). Interestingly, also a grafting density of 15 mol % PHEG increased MPS-uptake. However, the circulation times of liposomes grafted with 1, 2.5, 7.5, and 15 mol % PHEG did not differ significantly.

The results in this study show that coating the liposomes with poly(L-glutamine) with longer hydroxyalkyl side chains (PHPG and PHBG) instead of PHEG resulted in decreased circulation half-lives (Figure 4). An explanation for this observation may be found in the conformation of the polymers when dissolved in water. It has been suggested that suitable stealth polymers should be flexible and adopt a random conformation (31). E.g. dextran has a lower chain flexibility than PEG due to its carbohydrate backbone. When grafted on liposomes dextran is not able to prolong circulation times (32). Altschuler et al. showed that for poly(hydroxyalkyl-L-glutamine)s the degree of conformational freedom is optimal with hydroxyethyl as a side group and conformational freedom decreases for longer hydroxyalkyl groups. The use of hydroxypropyl and, even more so, hydroxybutyl resulted in the formation of α-helices in the polymer (33). The decrease in flexibility of these polymers correlates with the observed decrease of circulation half-life.

An important advantage of polymer-coated LCL over LCL without polymer-lipid conjugates is that incorporation of PEG leads to dose-independent pharmacokinetics over a broad dose range (34). To evaluate whether this phenomenon also applies to liposomes coated with poly(hydroxyalkyl L-amino acid)-lipid conjugates, pharmacokinetics of PHEA-liposomes were compared with PEG-liposomes at four different lipid doses ranging from

0.005 to 5 μmol total lipid per rat. The lowest dose level at which the PEG-liposomes appeared to show dose-independent kinetics was around 500 nmol/rat (Table III) which corresponds with earlier reported data (35). Interestingly, with PHEA-liposomes dose-dependency of the pharmacokinetics was less pronounced even at a 10-fold lower dose. PHEA-liposomes may therefore offer an additional advantage over PEG-liposomes in cases when very low lipid doses are preferred, such as in scintigraphic detection of sites of pathology (7).

The final aim of this study was to show that successful drug targeting is possible, with liposomes exposing biodegradable polypeptide-lipid conjugates. Within the scope of this thesis a PEG-liposomal formulation of prednisolone phosphate (PLP) was developed that was shown to efficiently contain the drug in the circulation and to highly effectively deliver the encapsulated drug to inflamed joints in experimental arthritis models (Chapter 3 and 4). Incorporation of PEG increased the circulation half-life of the liposomal formulation from 6 hrs to 18 hrs, which was shown to be necessary to achieve sufficient local delivery for complete reversal of the development of joint inflammation in these models (Chapter 3). The present study shows that the use of PHEA-liposomes as PLP carrier results in a similar pharmacokinetic profile of encapsulated PLP. Also, the therapeutic activity of 10 mg/kg PLP-PHEA-liposomes in rat adjuvant arthritis was equal to that of 10 mg/kg PLP-PEG-liposomes. In Chapter 3 and 4 we report that non-encapsulated PLP was inactive at this dose level. The observation that a dramatic improvement of the therapeutic effect of PLP can be achieved with PHEA-liposomes, clearly points to the drug targeting potential of this formulation.

In conclusion, we present a novel sterically stabilized liposome formulation performing similarly to PEG-liposomes regarding *in vivo* long-circulation behavior and drug targeting potential. These novel polymer-coated liposomes are the first that are sterically stabilized with a biodegradable polymer coating.

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CHAPTER 10

SUMMARIZING DISCUSSION

1. Introduction

Glucocorticoids are highly effective anti-inflammatory drugs that are applied in a range of different pathologies varying from local allergic reactions to severe, chronic inflammatory disorders (1). However, successful application in the clinic is limited due to a high incidence of serious adverse effects (2-4). Besides a poor safety profile, also poor localization in pathological target sites limits the usefulness of corticosteroids in the patient (5).

Targeted drug delivery with liposomal drug carriers that selectively accumulate at target tissues after i.v. injection may offer perspective (6). Targeted delivery may be considered a form of local treatment of all target sites by simply employing the systemic route (7,8). Chapter 1 presents the primary aim of the work described in this thesis: to design, evaluate and optimize long-circulating glucocorticoid-containing liposome formulations for the i.v. treatment of inflammatory disorders.

Liposomes are small lipid bilayer vesicles with an aqueous interior that can be used to entrap water-soluble glucocorticoid derivatives. By introducing specific functionalities to the bilayer, liposomes can acquire the ability to selectively accumulate at pathological target tissues, such as sites of inflammation or tumors. Such functionalities include ligands (e.g. antibodies) that specifically recognize receptors at cell surfaces in target tissues (active targeting). However, simple modifications such as polymer coatings that impose long-circulating behavior to liposomes can also introduce selectivity for the target site (passive targeting). Chapter 2 of this thesis provides an overview of both passive and active targeting strategies with liposomes.

The first part of the work, as described in Chapter 3, 4, 5 and 6 focuses on the therapeutic activity of long-circulating glucocorticoid liposomes in a series of different experimental animal models. The second part, described in Chapter 7 and 8 and 9 addresses the safety issues of liposomal glucocorticoid and approaches are proposed for further optimization of the formulation.

2. Therapeutic activity of liposomal glucocorticoid

2.1 Rheumatoid arthritis

In this thesis, the primary focus was directed towards the treatment of arthritis. Rheumatoid arthritis is a chronic autoimmune disorder, involving joint inflammation and progressive cartilage destruction (9). Rheumatoid arthritis is primarily an inflammatory disease of the connective tissue characterized by spontaneous remissions and exacerbations (flare-ups). Glucocorticoids can be highly effective in treating joint inflammation, but systemic application is only reserved for severe, refractive forms of inflammatory diseases in which other drugs are not sufficiently active (10). In rheumatoid arthritis local treatment can be realized by means of intra-articular injection. Nevertheless, this treatment approach is only attractive when single large joints are affected. In many cases rheumatoid arthritis involves a range of (smaller) affected joints and systemic therapy remains the only option (11).

In Chapter 3 of this thesis the therapeutic response to prednisolone phosphate encapsulated in long-circulating PEG-liposomes in rat adjuvant arthritis was evaluated. The results indicate that in this experimental arthritis model a single i.v. injection of 10 mg/kg prednisolone phosphate-PEG-liposomes can already induce a strong, rapid and long-lasting therapeutic effect. Complete remission of joint inflammation could be accomplished within two days of treatment, and the therapeutic benefit of the injection lasted for up to two weeks. Free prednisolone phosphate was not effective. Limited efficacy was observed only with a treatment regimen involving daily injections of free glucocorticoid. The results in this Chapter also show that the increased therapeutic activity results from drug targeting, as equal doses of prednisolone phosphate encapsulated in liposome types that home to the inflamed joint to a lesser extent also produce a weaker therapeutic response.

Roughly the same results were obtained in the murine collagen induced arthritis model, as described in Chapter 4. A single injection of 10 mg/kg liposomal prednisolone phosphate proved to be sufficient for complete, long-lasting remission of joint inflammation, whereas free prednisolone phosphate could slightly inhibit the progression of the inflammation reaction only when given daily during 5 days. The therapeutic activity of 5 times 10 mg/kg free prednisolone phosphate roughly corresponded to the effect of a single dose of 1 mg/kg liposomal prednisolone phosphate, illustrating the dramatic increase of the therapeutic effect conferred by liposomal encapsulation. Although 1 week post-treatment joint inflammation clearly recurred, cartilage erosion was still reduced in mice treated with prednisolone phosphate-PEG-liposomes, as witnessed by histological inspection of knee joint slides. Apparently, treatment with liposomal prednisolone phosphate resulted in a profound delay of the cartilage erosion process.

The results presented in both chapters provide promising prospects for a novel treatment approach in rheumatoid arthritis. The fact that a single dose efficiently affects all inflamed sites for a prolonged period of time may not only improve the therapeutic benefit and safety of i.v. glucocorticoids, it may also be more patient friendly. It can be envisaged that a single i.v. dose of liposomal glucocorticoid could be sufficient for overcoming an exacerbation of joint inflammation. The finding that cartilage erosion could be significantly delayed by targeted delivery of liposomal glucocorticoid may be of importance as glucocorticoid treatment of rheumatoid arthritis is often criticized for the lack of ability to induce a delay in the progression of joint erosion (12,13).

2.2 Multiple sclerosis

Like rheumatoid arthritis, multiple sclerosis is also an autoimmune disease involving inflammation and progressive tissue degeneration, in this case in the central nervous system (14). Relapses frequently occur and attenuation of these active stages of the disease is currently realized with intravenous high doses of glucocorticoids (1 or 2 grams methylprednisolone or prednisolone, daily for 3 – 5 days) (15). There is a strong need for the development of new therapies, as for relapses of multple sclerosis there is no treatment with a proven long-term effect available.

In Chapter 5 we describe the therapeutic effect of liposomal glucocorticoid formulations in two rat models of experimental encephalomyelitis. Like in experimental arthritis the results showed that long-circulating liposomes selectively enter the inflamed extravascular tissue in the spinal chord, which results in a higher local concentration of glucocorticoid. The improved target localization of liposomal glucocorticoid resulted in increased rate of T cell apoptosis and reduced infiltration of T cells and macrophages and completely resolved the disease-induced damage of the blood brain barrier. Furthermore, clinical signs such as paralysis of the limbs and tail were significantly reversed, which was not observed with free glucocorticoid treatment.

These data suggest that the added therapeutic benefit of liposomal encapsulation also applies to glucocorticoid treatment of inflammatory neurological disorders. Not only a superior anti-inflammatory effect but also significant benefit at the level of tissue destruction is achieved with liposomal glucocorticoid.

2.3 Oncology

Whereas surgery and radiation therapy can be successfully applied to treat patients with isolated primary and secondary tumors, in many cases chemotherapy is needed to eliminate metastases and/or non-resectable tumor tissue. Chemotherapy involves treatment with highly (cyto)toxic agents that pose a significant threat to healthy organs. An important limitation to the success of the current anti-cancer drugs is therefore the dose-limiting toxicity (16,17).

For the same reason as in inflammatory disorders, long-circulating liposomes may offer perspective. Besides sites of inflammation, also tumors show vasculature with enhanced permeability, enabling long-circulating liposomes to selectively localize in the extravascular tumor tissue (18). Studies with doxorubicin, daunorubicin, cisplatin and taxol showed that encapsulation in long-circulating liposomes can result in enhanced and prolonged exposure of the tumor to the drug and clearly improved the therapeutic outcome (18-21). Nevertheless, treatment with cytotoxic agents implies a range of long-term risks such as mutagenicity and teratogenicity, also with liposomally encapsulated cytotoxic drugs. Furthermore, liposomal encapsulation may lead to new forms of toxicities, such as the induction of skin reactions by liposomal doxorubicin (22). A lot of attention has therefore been paid to therapeutic agents without direct cytotoxic activity that realize an indirect anti-tumor effect by affecting (patho)physiological processes required for tumor growth. Current studies tend to focus on agents that intervene at the level of the blood supply and angiogenesis (neovascularization) in the tumor (23).

Glucocorticoids also belong to the group of non-cytotoxic agents shown to be able to inhibit angiogenesis. However, to become active against tumors, extremely high and frequent doses are necessary. Such doses were shown to induce morbidity and mortality in laboratory animals (24,25). In Chapter 6 we show that at a single dose level of 20 mg/kg, strong anti-tumor activity could be realized in two murine models of solid tumors. Tumor growth was almost fully inhibited by weekly injections of liposomal glucocorticoid.

Furthermore, tumor histology showed many interesting signs of strong beneficial effects, like massive apoptosis of tumor cells, obstruction of the tumor vascularization and encapsulation by fibrous tissue and isolation of the tumor nodule from healthy tissue.

Either alone or in combination therapy, liposomal glucocorticoid may show high therapeutic value in cancer treatment, potentially offering the advantage of strong anti-tumor activity without the acute and long-term side effects that are usually associated with chemotherapy.

3. Mechanism of action

3.1 Pharmacokinetics and localization at the target site

The observation that liposomal encapsulation could lead to such a strong increase of the therapeutic activity of glucocorticoids was unexpected. In fact, it shows that the currently employed systemic treatment approach with free glucocorticoid may be highly inefficient. In free form, glucocorticoids show a large volume of distribution and a high clearance rate (5). The fraction of the injected dose that will actually reach the target site is therefore low and the majority of the administered drug may be either prematurely eliminated from the body or localize at healthy non-target tissues. The results in adjuvant arthritis rats (Chapter 3) clearly show the consequences of liposomal encapsulation for the pharmacokinetics of glucocorticoids. Estimations based on the plasma concentration-time curves of free and liposomal prednisolone phosphate shown in this chapter suggest that liposomal encapsulation can decrease the distribution volume in a rat from more than a liter to approximately 10 ml, which roughly equals the plasma volume. Also, the clearance rate is reduced from approximately 2 $L \cdot h^{-1} kg^{-1}$ to less than 2 $ml \cdot h^{-1} kg^{-1}$.

In itself altered pharmacokinetic behavior does not imply increased target localization and/or enhanced therapeutic activity. Two additional requirements must be met: (1) the target site must be directly accessible for the liposomes residing in the circulation, and (2) as the liposomally encapsulated phosphate ester of the glucocorticoid is not therapeutically active, it must be released from the liposome and converted to the active form of the drug.

1) Access to the target site. In rat experimental arthritis (Chapter 3), experimental encephalomyelitis (Chapter 5) and murine experimental tumors (Chapter 6) the organ distribution data of radioactively labeled liposomes clearly point to preferential accumulation at pathological sites. Also the data with gold-labeled liposomes show that liposome localization is clearly inflammation-driven. Both in murine experimental arthritis as well as in experimental encephalomyelitis gold is present at the inflamed sites whereas healthy tissues are largely devoid of gold. It has been suggested that liposomes localize at pathological tissues as a result of uptake by infiltrating monocytes/macrophages (26). However, in our case, the radioactivity that was found in the cell fraction after centrifugation of the blood was minimal. Therefore, it appears more likely that liposomes selectively localize at

inflamed/malignant sites as a result of enhanced vascular permeability. This phenomenon has been studied and described in many previous publications (26,27).

2) Release of the encapsulated drug. The microscopic tissue slides obtained with gold-labeled liposomes in experimental arthritis (Chapter 4) and experimental encephalomyelitis (Chapter 5) point out that the liposomes become cell-associated after localization in the pathological target site. In both cases resident macrophages appear to take up the majority of the liposomes. Therefore, it seems likely that liposomal glucocorticoid ends up in the intracellular endosomal/lysosomal compartment. Liposomal glucocorticoid may be released by enzymatic degradation of the liposome and subsequently converted from the non-active phosphate form into the free (active) glucocorticoid. Free glucocorticoid is able to freely cross cellular membranes, enabling the drug to reach the cytoplasm of the macrophage and possibly also to the extracellular space. Such a macrophage-mediated release process has been described earlier for liposomal doxorubicin and liposomal 5-fluoro-2'-deoxyuridine (28,29).

3.2 Role of macrophages at the target site

In arthritis an important role in the pathology of arthritis is ascribed to macrophages present in the synovial lining layer that surrounds the connective tissue in the joints. They have been shown to produce many pro-inflammatory cytokines, attract new inflammatory cells and produce enzymes that can damage the cartilage (30,31). Generally, these functions can be effectively suppressed by glucocorticoids. Glucocorticoids bind to the cytosolic glucocorticoid receptor, which can then translocate to the nucleus where it directly modulates DNA transcription of a variety of genes that encode for pro-inflammatory cytokines or tissue-degenerating enzymes (32,33). GC also exert rapid non-genomic effects. These are thought to occur by intracellular signaling via second messengers (like protein kinases and cyclic AMP) upon binding to GC receptors, non-specific interactions with cellular membranes, or specific interactions with membrane bound GC-receptors (34).

The observation that especially the macrophages in the synovial lining very actively internalized liposomes alerted our interest. The high intracellular concentrations of glucocorticoid are likely to account for a strong suppressive effect on synovial macrophages leading to amelioration of inflammation and reduction of joint erosion. Furthermore, as free glucocorticoid can easily pass cellular membranes, the drug may escape from the intracellular compartment and be released into the extracellular environment, also suppressing other cells in the synovium, such as T cells or endothelial cells.

In experimental encephalomyelitis, liposome uptake by macrophages in the inflamed areas was also observed. Besides a direct suppressive effect on macrophages, as illustrated by reduced TNF-α production, liposomal glucocorticoid enhanced apoptosis of T cells, indicating that free glucocorticoid can indeed escape from macrophages affecting other cells at the inflamed site. The observed reduction of leukocyte infiltration may be a result of down-regulation of the production of chemoattractants by macrophages and/or resulting from the restoration of the integrity of the blood-brain-barrier. Also, localization of glucocorticoid

liposomes in peripheral immune organs leads to a reduction of the number of circulating leukocytes. The reduced infiltration of macrophages was only seen with liposomally encapsulated glucocorticoid. Interestingly, this may help to prevent ongoing tissue destruction and thereby exert beneficial long-term effects on the disease course.

Macrophages are known to also play a role in tumor growth. As the anti-tumor effect we observed with liposomal glucocorticoid proved to be indirect (no tumorcidal activity of glucocorticoid was noted in *in vitro* tumor cell cultures), it may be explained by the strong suppressive effect of liposomal glucocorticoid on activated macrophages. Several investigators report that tumor-associated macrophages indirectly promote tumor growth by either secreting pro-inflammatory cytokines that induce angiogenesis and/or producing tissue-degrading enzymes, such as matrix metalloproteinases, that enable the tumor to invade into healthy tissue. Tumors appear therefore to be dependent to some degree on the infiltration of macrophages for their own growth and survival (35,36). Down regulation of the production of proangiogenic factors and tissue-degrading enzymes by macrophages with liposomal glucocorticoid may be an attractive strategy to suppress tumor growth without the use of cytotoxic agents.

4. Safety aspects of liposomal glucocorticoid

4.1 Systemic adverse effects

In addition to the affinity of liposomal glucocorticoid for macrophages at sites of pathology also macrophages in organs that are part of the mononuclear phagocyte system, such as liver, spleen and bone marrow, extensively take up liposomes. The fact that these macrophages are loaded with glucocorticoid may have two important consequences that relate to toxicity: first, liposomal glucocorticoid may be expected to be converted into free, active glucocorticoid entering the circulation and second, cellular functioning of these macrophages may be (temporarily) suppressed. The first phenomenon may result in systemic (adverse) effects of free glucocorticoid and is discussed below. The second aspect deals with the concern of unwanted suppression of the body's immune defense against blood-born pathogens and is discussed under 3.2.

The results presented in Chapter 7 reveal that adverse effects that are typical for systemic glucocorticoid treatment, such as body weight loss and hyperglycemia, can also occur upon injection of liposomal glucocorticoid (37,38). Interestingly, these adverse effects were observed with free and liposomal dexamethasone, but not with liposomal prednisolone. These results corresponded with the observation that upon injection of liposomal dexamethasone phosphate relatively high levels of free glucocorticoid in the circulation were observed, much higher than in the case of liposomal prednisolone phosphate. As both liposome formulations were shown to be non-leaky in the circulation, we hypothesize that the 20 – 30% of the injected dose of liposomal drug that localized in liver and spleen may have been taken up by MPS macrophages, degraded, and subsequently released by the

macrophages as free drug back into the circulation (28,29). The observation that the plasma levels of free glucocorticoid after injection of liposomal dexamethasone phosphate were considerably higher than after liposomal prednisolone phosphate is likely the result of a higher clearance rate of prednisolone as compared to dexamethasone. In fact, the relatively high clearance rate of prednisolone in rats may have been the critical parameter preventing pharmacologically active plasma levels and corresponding systemic adverse effects.

As a high clearance rate of the free glucocorticoid in the circulation appears to be crucial to optimize the therapeutic index of liposomal glucocorticoid, the group of inhaled/intranasal glucocorticoids may be of high interest. The success of these glucocorticoids in topical treatment of asthma and rhinitis can largely be attributed to their high local activity in combination with the fact that the liver highly efficiently eliminates the fraction of administered drug entering the circulation after inhalation and ingestion (39,40). By means of targeted delivery with long-circulating liposomes, patients with inflammatory disorders such as rheumatoid arthritis or multiple sclerosis may also benefit from this group of glucocorticoids. Data provided in Chapter 7 confirm the value of such an approach with budesonide as a representative of this group.

4.2 Suppression of MPS-function

The second type of potential adverse effects investigated is also related to the encapsulated glucocorticoid, but differs from the effects discussed above in the sense that it is not observed with free drug. The fact that liposomally encapsulated glucocorticoids cannot easily enter healthy tissues may reduce a range of adverse effects, but it should be realized that a significant fraction of the injected dose ends up in the liver, spleen and bone marrow. The majority of this fraction is taken up by macrophages and the latter cells may therefore become at risk for adverse effects induced by liposomally encapsulated glucocorticoid (41). As the MPS has an important function as a part of the immune system's first line of defense against pathogens in the circulation, suppression of macrophages by liposomally encapsulated glucocorticoid may result in enhanced susceptibility for infections.

To evaluate the extent to which liposomal glucocorticoid can suppress the phagocytic capacity of liver macrophages, blood clearance of bacteria was monitored after injection of $5 * 10^5$ *K Pneumonia*, 24 hrs after rats were pretreated with 10 mg/kg liposomal prednisolone phosphate and compared to control treatments (saline, free prednisolone phosphate, and empty liposomes). At 60 and 120 min after i.v. injection of the bacteria, blood was collected in which the number of viable bacteria was determined.

At 2 hrs after injection of the bacteria, all control treatments (saline, free prednisolone phosphate and empty liposomes) showed 99.98 % removal of bacteria from the circulation, whereas after treatment with liposomal prednisolone phosphate 99.8% of the bacteria was removed ($p<0.05$, One way ANOVA). This result suggests that liposomal glucocorticoid can affect the clearance capacity of MPS macrophages. However it should be realized that *K Pneumonia* is a highly encapsulated bacterial strain for which optimal MPS-functioning is crucial. The fact that this is a highly sensitive test and that the majority of

bacteria is still rapidly cleared from the circulation may reduce concerns regarding MPS dysfunction. Dose escalation studies in patients are necessary to reveal whether suppression of MPS-macrophages will become a dose limiting form of toxicity.

4.3 Complement-related hypersensitivity reactions

Chapter 8 addresses a third type of potential adverse effects, not related to the glucocorticoid but rather a result of the liposomal carrier itself. Several studies revealed that liposomes, in particular PEG-liposomes, can cause hypersensitivity reactions in 5-10% of the patients upon i.v. injection. Although these reactions are generally mild and short lasting, they caused some turbulence in the liposome field as i.v. administration of long-circulating liposomes was thought to be safe within a broad dose range (43,44). Mechanistic studies showed that these hypersensitivity reactions are most likely a result of the activation of complement (45,46).

In our study different liposome types were evaluated in an *in vitro* complement activation assay in human plasma and an *in vivo* pig model in which cardiopulmonary effects can be detected. All liposomes types tested caused complement activation and hypersensitivity reactions. Only a small-sized non-PEG-liposome (<70 nm) composed of rigid, neutral bilayers, lacked both complement activation and hypersensitivity reactions. As the same liposome type but with a mean size of 90 nm did induce reactions, to a similar extent as PEG-liposomes of approximately 90 nm, size seems to be an important additional parameter in complement activation besides composition-related aspects.

As we were not able to size the PEG-liposomes down to <70 nm, it was decided to evaluate the small non-PEG-liposomes regarding their applicability for passive drug targeting. To our surprise, the small non-PEG-liposome type appeared to have a longer circulation time than the 90 nm PEG-liposomes, which have been used for most of the work presented in this thesis. Evaluation of glucocorticoids encapsulated in this type of liposome in experimental arthritis revealed a comparable therapeutic effect, indicating that glucocorticoid encapsulated in small, neutral non-PEG-liposomes may be preferred over glucocorticoid-PEG-liposomes in the clinic.

5. Further development of glucocorticoid formulations

5.1 Design of novel long-circulating liposome compositions

Chapter 9 describes the development of novel liposome coating polymers, other than PEG and based on L-amino acids as monomers. With both poly(hydroxyethyl-L-glutamine) and poly(hydroxyethyl-L-asparagine) liposomes could be prepared that were as long-circulating as PEG-liposomes and also as effective as PEG-liposomes in targeted delivery of glucocorticoids to sites of inflammation in experimental arthritis. These liposomes are novel in that they are coated with biodegradable polymers that can be degraded by proteolytic enzymes, which may be abundant at sites of pathology. Interestingly, liposomes coated with

poly(hydroxyethyl-L-asparagine) could relatively easily be downsized to approximately 50 nm, which was not possible with PEG-liposomes. This may offer perspective regarding the avoidance hypersensitivity reactions, as size appeared to be a crucial parameter to be taken into account in liposome-induced complement activation.

5.2 Selection and encapsulation of new glucocorticoids

We found that from the glucocorticoid, which is encapsulated in the aqueous interior, the water-soluble phosphate ester is required for stable encapsulation. Specific examples of water-soluble glucocorticoids that can be used in the liposomal approach are prednisolone disodium phosphate, dexamethasone disodium phosphate, betamethasone disodium phosphate, and triamcinolone acetate disodium phosphate. To minimize the systemic effects, the use of topical glucocorticoids with a high clearance rate seems advantageous, as shown by the work in this thesis. Success was booked with a newly synthesized phosphate ester of the asthma glucocorticoid budesonide.

Two other interesting approaches for further optimization of liposomal glucocorticoid deserve attention. Recent research has revealed that certain glucocorticoids have a surprisingly potent direct effect on inflammatory cells that does not involve down-regulation of cytokine production at the level of gene transcription. Rather, this phenomenon is mediated at a non-genomic level and appears to be of importance when a high concentration of glucocorticoid is present at sites of inflammation (34). From this perspective, methylprednisolone, which is currently applied in high dose i.v. treatment of relapses of multiple sclerosis and rheumatoid arthritis, appears to be an interesting candidate for evaluation in the concept of targeted delivery by long-circulating liposomes.

A second group of glucocorticoids of high interest are the 11-keto glucocorticoids. These glucocorticoids are in principle inactive and require a specific enzyme (11ß-hydroxysteroid dehydrogenase) to be converted to the active form. As relatively high quantities of this enzyme are present selectively at sites of inflammation, incorporation of these glucocorticoids could lead to additional target specificity (47,48). An interesting candidate for encapsulation in long-circulating liposomes is prednisone. In future research both the above-mentioned approaches will be evaluated.

Typically the hydration-extrusion method of preparation results in encapsulation of 5-10% of the glucocorticoid inside the liposomes. The formulation can be stored at 4 °C and remains stable for at least 1 year (less than 5% leakage and less than 5% increase of particle size). To further improve the encapsulation efficiency and long-term stability, at the moment an improved preparation method is being developed, using a 'remote-loading' technique. Up to 100 % of the added glucocorticoid can potentially be encapsulated by using this method, resulting in reduced loss of glucocorticoid during manufacturing of the preparation. Evaluation of such preparations in experimental arthritis revealed that remote loading does not result in loss of therapeutic activity.

Besides increased encapsulation efficiency, remote loading often enables successful lyophilization of liposomally encapsulated drugs, which increases the long-term stability of the product. Whether this also applies to remote-loaded liposomal glucocorticoid remains to be evaluated. All together these additional developments must lead to a product that besides strong therapeutic activity shows optimal safety in humans, with attractive properties regarding (large-scale) production and long-term storage.

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APPENDICES

SAMENVATTING IN HET NEDERLANDS

1. Inleiding

Glucocorticoïden zijn zeer effectieve ontstekingsremmende geneesmiddelen die kunnen worden toegepast bij een breed scala aan ontstekingsziekten, variërend van kleinschalige allergische reacties tot ernstige inflammatoire aandoeningen zoals reuma, de ziekte van Crohn en multiple sclerose. Een groot probleem bij behandeling met glucocorticoïden is dat deze stoffen zich na toediening snel over het gehele lichaam verspreiden, veel gezonde organen bereiken en zich slechts in beperkte mate concentreren in de ontstekingshaarden. Dit ongunstige gedrag maakt het noodzakelijk dat de dosis omhoog moet om de gewenste therapeutische werking te verkrijgen. Dat is niet alleen verspilling van de toegediende glucocorticoïden, het is met name ook een belangrijke oorzaak van allerlei (vaak ernstige) neveneffecten.

Een belangrijke vooruitgang werd geboekt met de introductie van lokale behandelingsstrategieën, waarmee zeer effectief ziekten zoals bijvoorbeeld astma en allergische huidreacties kunnen worden bestreden zonder dat de toegediende glucocorticoïden terecht komen op gezonde plaatsten waardoor ongewenste neveneffecten kunnen ontstaan. Helaas kunnen veel ontstekingsziekten moeilijk worden behandeld door middel van lokale toediening van glucocorticoïden, omdat de ontstekingshaarden slecht toegankelijk zijn en/of te veel verspreid zijn over het lichaam. Gestuurde aflevering van glucocorticoïden door liposomale geneesmiddeldragers die in de ontstoken plekken in het lichaam ophopen (‘*targeting*’) zou uitkomst kunnen bieden. *Targeting* kan zo worden beschouwd als een vorm van lokale behandeling van alle ontstekingen in het lichaam eenvoudigweg door de liposomen in de bloedbaan te injecteren (‘intraveneuze’ toediening).

Liposomen zijn minuscule vetblaasjes ter grootte van een duizendste tot een tienduizendste van een millimeter, waarin geneeskrachtige stoffen, zo ook glucocorticoïden, kunnen worden ingesloten. Liposomen worden veelal gemaakt door bepaalde vetten te dispergeren in een waterige oplossing van het geneesmiddel. De gewenste afmeting van de vetblaasjes die op deze wijze ontstaan wordt verkregen door de dispersie meerdere keren door filtermembranen te persen met de juiste poriegrootte. Door het oppervlak van de vetblaasjes te modificeren met stoffen die affiniteit vertonen met het aangedane weefsel, kan *targeting* met de liposomen gerealiseerd worden. Als hierbij gebruik wordt gemaakt van biologische stoffen zoals eiwitten en suikerverbindingen die specifieke interacties aangaan met zieke cellen op de aangedane plek, spreekt men van ‘actief gestuurd geneesmiddeltransport’ (‘*active drug targeting*’ in het Engels).

Echter, soms kunnen synthetische stoffen zoals polymeren er al voor zorgen dat liposomen affiniteit krijgen met de aangedane plekken in het lichaam. Maar dit gebeurt dan eigenlijk op een indirecte manier. Normaal gesproken worden liposomen, net als bijvoorbeeld bacteriën en andere indringers, uit de bloedbaan verwijderd door macrofagen. Macrofagen zijn belangrijke cellen van het immuunsysteem, die zich onder andere in grote getale in de

lever en de milt bevinden. Polymeren op het oppervlak kunnen dit proces echter belemmeren. De liposomen worden dan 'langcirculerend' en krijgen daardoor beter de kans om langzaam op te hopen op zieke plekken in het lichaam, omdat de bloedvaatwanden op die plekken doorlaatbaar zijn. Het voor dit doel meest gebruikte langcirculerende liposoom zijn de zogenaamde 'PEG-liposomen', die gemodificeerd zijn met het polymeer polyethyleenglycol (PEG). Deze indirecte manier van het bereiken van de gewenste plaatsen wordt ook wel 'passief gestuurd geneesmiddeltransport' genoemd ('*passive drug targeting*'). In Hoofdstuk 2 worden de varianten en mogelijkheden van *active drug targeting* en *passive drug targeting* met liposomen op een rij gezet in een uitgebreid literatuuroverzicht.

Vooral *passive drug targeting* met langcirculerende liposomen lijkt aantrekkelijk om de effectiviteit van glucocorticoïden in ontstekingsziekten zoals reuma en multiple sclerose te verbeteren en de schadelijke neveneffecten te verminderen, omdat met langcirculerende liposomen het toegediende glucocorticoïde selectief naar de ontstekingshaarden kan worden gebracht. Het voornaamste doel van het onderzoek dat in dit proefschrift beschreven is, was dan ook het ontwerpen, evalueren en optimaliseren van langcirculerende glucocorticoïde-bevattende liposomen voor de intraveneuze behandeling van inflammatoire aandoeningen. Het eerste deel van het proefschrift, hoofdstuk 3 tot en met 6, richt zich op het therapeutische effect van langcirculerende glucocorticoïdebevattende liposomen in een aantal experimentele ziektemodellen in proefdieren. Het tweede deel omvat hoofdstuk 7 tot en met 9 waarin de mogelijke neveneffecten van liposomale glucocorticoïden worden belicht en mogelijkheden worden bestudeerd om dit nieuwe therapeutische preparaat verder te optimaliseren.

2. Therapeutisch effect van liposomaal glucocorticoïde

2.1 Reumatoïde artritis

Het onderzoek dat in dit proefschrift beschreven is richtte zich aanvankelijk grotendeels op reumatoïde artritis. Reumatoïde artritis, of kortweg reuma, is een chronische inflammatoire aandoening die zich manifesteert in de vorm van gewrichtsontstekingen en op den duur leidt tot onherstelbare gewrichtsschade. De gewrichtsontstekingen kunnen met verschillende geneesmiddelen worden bestreden, maar voor een snelle, effectieve onderdrukking van ernstige uitbarstingen van de ontstekingen ('exacerbaties') is men veelal aangewezen op glucocorticoïden. Dat glucocorticoïden vaak achter de hand gehouden worden als 'laatste redmiddel' moet worden toegeschreven aan het relatief ongunstige neveneffectenprofiel van deze geneesmiddelen na intraveneuze of orale toediening. Lokale toediening door injecties direct in het gewricht kan een enorme vooruitgang betekenen, maar met één injectie kan slechts één gewricht tegelijk worden behandeld terwijl er meestal meerdere gewrichten tegelijk zijn ontstoken.

In Hoofdstuk 3 van dit proefschrift wordt het therapeutische effect beschreven van het glucocorticoïde prednisolonfosfaat, verpakt in PEG-liposomen, bij een kunstmatige,

experimentele vorm van artritis in ratten, het zogenaamde ‘rat adjuvant artritis’-model. De resultaten wezen op een snel, sterk en langdurig effect dat al gerealiseerd kon worden met één enkelvoudige injectie (dosis: 10 mg/kg). Binnen twee dagen na de behandeling waren alle gewrichtsontstekingen volledig verdwenen en het therapeutisch voordeel bleef zichtbaar tot twee weken na behandeling. Prednisolonfosfaat in vrije vorm bleek niet effectief. Pas na dagelijkse toediening van het vrije glucocorticoïde over een periode van zeven dagen werd een matig effect geobserveerd. Vervolgens werd bekeken of deze drastische verbetering inderdaad het resultaat was van *passive drug tageting*. Dit kon worden bevestigd na de waarneming dat prednisolonfosfaat in liposoomtypen, die in mindere mate terechtkomen in ontstoken gewrichten, ook minder therapeutisch actief zijn.

Vergelijkbare resultaten werden gevonden bij een andere experimentele vorm van artritis: het ‘collageen type II-geïnduceerde artritis’-model in muizen. Hoofdstuk 4 laat zien dat met een enkelvoudige dosis, vergelijkbaar aan die in rat adjuvant artritis, ook in deze vorm van artritis een volledige en langdurige remissie van de gewrichtsontstekingen kon worden bewerkstelligd. Prednisolonfosfaat in vrije vorm was alleen beperkt effectief bij dagelijkse injecties van vergelijkbare doses. Het effect van vijf maal dagelijks vrij prednisolonfosfaat bleek vergelijkbaar met het effect van één enkele – tien maal lagere – dosis liposomaal prednisolonfosfaat, hetgeen goed de sterke geneeskrachtige werking van het liposomale preparaat illustreert. Uit microscopische bestudering van kniegewrichten van muizen die behandeld waren met goud-gemarkeerde liposomen bleek heel duidelijk dat de liposomen in hoge mate worden opgenomen door actieve ontstekingscellen in de gewrichten. Ook bleek dat de kraakbeenschade in het gewricht sterk kan worden verminderd met de behandeling met liposomaal prednisolonfosfaat.

De resultaten in de hoofdstukken 3 en 4 lijken aan te geven dat liposomaal glucocorticoïde veelbelovend kan zijn voor de behandeling van reumapatiënten. Dat met een enkele injectie voldoende geneesmiddel kan worden afgeleverd voor een langdurig effect in alle ontstoken gewrichten, betekent niet alleen dat een groter therapeutisch voordeel met minder neveneffecten kan worden verwacht, maar dat deze behandelingsstrategie tevens minder belastend kan zijn voor de patiënt. Immers, het onderzoek suggereert dat een eenmalige behandeling voldoende is om een exacerbatie van gewrichtsontstekingen volledig te bestrijden. Dat ook de gewrichtsschade lijkt te worden vertraagd door behandeling met liposomaal glucocorticoïde is belangrijk omdat de behandeling van reuma met glucocorticoïden vaak bekritiseerd wordt vanwege het gebrek aan vermogen van glucocorticoïden om naast de ontsteking ook de toenemende gewrichtsschade een halt toe te roepen.

2.2 Multiple sclerose

Multiple sclerose (kortweg ‘MS’) is net als reumatoïde artritis een inflammatoire aandoening. Bij MS komen de ontstekingen voornamelijk voor in het centrale zenuwstelsel. Daar leiden ze tot toenemende schade aan en uitval van zenuwcellen zodat er langzaam maar zeker steeds meer verlammingsverschijnselen optreden gedurende het ziekteverloop.

Voor de behandeling van MS zijn nog maar heel weinig adequate geneesmiddelen voorhanden. Regelmatig worden intraveneus hoge doseringen glucocorticoïden gegeven (1 tot 2 gram methylprednisolon dagelijks gedurende drie tot vijf dagen) om de ontstekingsreacties enigszins te kunnen onderdrukken. In Hoofdstuk 5 wordt het therapeutisch effect beschreven van liposomaal glucocorticoïde in een experimenteel diermodel van MS. Net als bij experimentele reuma liet ook dit diermodel zien dat de liposomen selectief ophopen in ontstoken weefsel, in dit geval het ruggenmerg. Bovendien werden ook in dit model de goud-gemarkeerde liposomen ter plaatse teruggevonden in de macrofagen, de immuuncellen die mede verantwoordelijk worden gehouden voor de ontstekingen. De verhoogde concentraties glucocorticoïde die zo door de liposomen in de ontstekingshaarden worden opgebouwd, leidden tot uitschakeling en verlaagde infiltratie van ontstekingscellen en tot een volledig herstel van de beschadigde bloed-hersenbarrière, het vlies dat het centraal zenuwstelsel scheidt van het bloedvaatstelsel. Bovendien werden de verlammingsverschijnselen bij de dieren verminderd, hetgeen niet kon worden bewerkstelligd met hoge doses vrij glucocorticoïde.

Deze resultaten suggereren dat het therapeutische voordeel dat kan worden geboekt door insluiting van glucocorticoïden in langcirculerende liposomen van toepassing is voor meerdere inflammatoire aandoeningen. Indien er sprake is van ontstekingshaarden waar liposomen in kunnen ophopen vanuit de bloedbaan valt er therapeutisch voordeel te verwachten van glucocorticoïden in langcirculerende liposomen. Dit betekent dat ook inflammatoire darmaandoeningen zoals colitis ulcerosa of de ziekte van Crohn of huidaandoeningen zoals psoriasis in aanmerking zouden kunnen komen voor behandeling met liposomaal glucocorticoïde. Of dit inderdaad zo is zal moeten blijken uit nader experimenteel onderzoek.

2.3 Toepassing in de oncologie

Operatieve verwijdering of behandeling door bestraling kunnen succesvol zijn bij de patiënten die lijden aan vormen van kanker waarbij sprake is van geïsoleerde, toegankelijke tumoren. Helaas zijn veel tumoren moeilijk verwijderbaar of zijn er al uitzaaiingen geconstateerd. In die gevallen is de patiënt meestal aangewezen op chemotherapie. Chemotherapie behelst de behandeling met de zeer giftige ‘cytostatica’, stoffen die aangrijpen op sneldelende cellen. Het belangrijkste probleem van chemotherapie is dan ook de schadelijkheid voor gezond weefsel, waardoor maar beperkte hoeveelheden cytostatica toegediend kunnen worden, met vaak slechts een beperkte anti-kankerwerking als gevolg.

Om dezelfde reden als bij inflammatoire aandoeningen kunnen langcirculerende liposomen mogelijk uitkomst bieden. Net zoals bij ontstekingen is er bij tumoren vaak sprake van verhoogde vaatwandpermeabiliteit zodat zich in het bloed bevindende liposomen selectief kunnen uittreden en het tumorweefsel kunnen bereiken. Studies met cytostatica als doxorubicine, cisplatina en taxol hebben laten zien dat insluiting in langcirculerende liposomen leidt tot verhoogde en verlengde blootstelling van de tumor aan het ingesloten geneesmiddel en dat dit resulteert in een verbeterd effect. Desondanks blijft de behandeling

met cytotoxische stoffen bedreigingen op de lange termijn met zich meebrengen, zoals mutageniciteit (afwijkingen in het genetisch materiaal van gezonde cellen) en teratogeniciteit (genetische afwijkingen bij het nageslacht). Daarom besteedt men veel aandacht aan geneesmiddelen die op een andere manier bij kanker werken dan cytostatica. Zo worden momenteel stoffen onderzocht die aangrijpen op de bloedvoorziening van de tumor en zo op een indirecte wijze de tumorgroei kunnen remmen.

Glucocorticoïden horen ook bij de groep geneesmiddelen die de bloedvoorziening van tumoren kunnen aantasten. Alleen wordt dit effect slechts gezien bij zeer hoge doseringen die frequent toegediend dienen te worden en ronduit levensbedreigend kunnen zijn bij klinische toepassing. In Hoofdstuk 6 laten we in experimentele tumormodellen bij muizen echter zien dat, met een enkelvoudige behandeling met een gangbare dosis glucocorticoïde, ingesloten in langcirculerende liposomen, al een sterk anti-tumor effect kon worden bewerkstelligd. Naast een vrijwel volledige remming van de tumorgroei werden ook op microscopisch niveau interessante verschijnselen in de tumoren waargenomen, zoals massale uitval van tumorcellen, obstructies in het tumorvaatbed en inkapseling van de tumor in bindweefsel zodat als het ware de tumor 'los' komt te liggen van het gezonde weefsel. Al met al zou deze behandelingsstrategie van grote waarde kunnen zijn, als een op zichzelf staande behandeling of in combinatie met operatieve verwijdering of chemotherapie. Toekomstig onderzoek zal moeten uitwijzen bij welke vormen van kanker liposomaal glucocorticoïde succesvol kan zijn.

3. Neveneffecten van liposomaal glucocorticoïde

3.1 Neveneffecten van het ingesloten glucocorticoïde

Hoewel met langcirculerende liposomen het ingesloten glucocorticoïde op gerichte wijze naar de aangetaste plaatsen gestuurd wordt, betekent dit zeker niet dat er helemaal geen glucocorticoïde meer in gezond weefsel terechtkomt. Naast ontstekingen en maligniteiten nemen ook organen als lever, milt en beenmerg liposomen op. Dit wordt veroorzaakt doordat zich in deze organen macrofagen bevinden, die gespecialiseerd zijn in het verwijderen van ongewenste indringers. Als deze macrofagen glucocorticoïde-bevattende liposomen opnemen uit de bloedbaan zou dit hun functioneren kunnen aantasten, omdat glucocorticoïden in het algemeen een remmend effect hebben op cellen van het immuunsysteem. Dit type neveneffect wordt niet beschreven in een van de hoofdstukken van dit proefschrift, maar is wel onderzocht. Voorlopige resultaten wijzen erop dat dit risico inderdaad bestaat, maar het onderdrukkende effect blijft zeer beperkt en meer dan 99% van de capaciteit van macrofagen om ziekteverwekkende indringers te elimineren blijft onaangetast.

Bovendien kan het ingesloten glucocorticoïde na opname door macrofagen uit deze cellen ontsnappen en opnieuw in de bloedbaan terecht komen maar dan in vrije vorm. Op die wijze kan het zich alsnog over het lichaam verspreiden en neveneffecten veroorzaken als ware het in vrije vorm toegediend. Voorbeelden van neveneffecten van glucocorticoïden in

vrije vorm zijn onder andere verhoging van het bloedsuikergehalte en veranderingen in lichaamsgewicht. Deze worden 'systemische' neveneffecten genoemd omdat ze in principe het lichaam in zijn geheel betreffen. In Hoofdstuk 7 worden genoemde neveneffecten in ratten bestudeerd, zowel na toediening van vrij als van liposomaal glucocorticoïde. Het blijkt dat deze neveneffecten kunnen worden vermeden mits een speciaal type glucocorticoïde wordt verwerkt in de liposomen dat in vrije vorm snel uit de bloedbaan wordt verwijderd door de lever. Dit type glucocorticoïde wordt al met groot succes toegepast bij lokale behandeling van ontstekingen, zoals bijvoorbeeld in inhalatietherapie bij astma. Immers, mocht er ondanks de lokale toediening toch glucocorticoïde in staat zijn de bloedbaan te bereiken dan zal dit snel wordt geïnactiveerd. Welnu, *targeting* met langcirculerende liposomen kan in feite ook gezien worden als een manier van lokale behandeling. Het liposoom beschermt het glucocorticoïde tegen voortijdige afbraak *en route* naar de ontstekingen waar het zeer effectief zijn werk kan doen, terwijl de snelle inactivatie in de lever voorkomt dat eventueel in de bloedbaan vrijgekomen glucocorticoïde systemische neveneffecten kan veroorzaken.

3.2 Neveneffecten van de liposomale drager

Hoofdstuk 8 gaat in op een heel ander type neveneffect dat niet gerelateerd is aan het glucocorticoïde, maar wordt veroorzaakt door het liposoomdeeltje zelf. Het betreft een acute allergische reactie die ontstaat bij 5 tot 10% van de patiënten direct bij aanvang van de infusie van de liposomen en die zich uit in roodheid, benauwdheid en bloeddrukveranderingen. De reactie is meestal mild en verdwijnt snel als de infusie wordt gestopt. Vaak kan de reactie voorkomen worden door te starten met een voldoende lage infusiesnelheid. De reactie wordt in verband gebracht met het zogenaamde 'complementsysteem', een onderdeel van het immuunsysteem dat bestaat uit een serie plasma-eiwitten die in een soort kettingreactie elkaar onderling kunnen activeren en op die manier allerlei afweerreacties in het lichaam kunnen uitlokken.

Om er achter te komen welke componenten van de liposomen verantwoordelijk kunnen worden gehouden voor deze complementactivatie-reacties, bestudeerden we de activatie van het complementsysteem na toediening van verschillende liposoomtypen aan humane bloedmonsters. De overgevoeligheidsreacties zelf werden gemeten in varkens die, blijkens vorige studies, uitstekend kunnen fungeren als een proefdiermodel als het gaat om complement-gerelateerde overgevoeligheid. Het optreden van complementactivatie en complementgerelateerde overgevoeligheid bleek echter niet zozeer gerelateerd te zijn aan de samenstelling van de liposomen, maar eerder te maken te hebben met de grootte van de liposoomdeeltjes. Liposomen van zeer klein formaat, met een rigide vetlaag en zonder PEG op het oppervlak bleken zowel in de bloedmonsters als in varkens het enige liposoomtype te zijn dat geen complementactivatie veroorzaakte. Dit type liposomen bleek ook heel goed bruikbaar voor *targeting* van glucocorticoïden in ontstekingsziekten. Het lijkt dus mogelijk acute overgevoeligheidsreacties te reduceren door de afmeting van de liposomen te reduceren en de samenstelling nauwgezet te definiëren.

4. Verdere ontwikkelingen

4.1 Nieuwe liposoomtypen

Het meest gebruikte langcirculerende liposoomtype in de studies beschreven in dit proefschrift zijn de PEG-liposomen. Zoals aangegeven in de inleiding zorgt de PEG-laag op het oppervlak van dit liposoom voor de langcirculerende eigenschap doordat het de opname uit de bloedbaan door lever en milt sterk reduceert. Hoofdstuk 9 beschrijft de ontwikkeling van een nieuw type polymeren voor langcirculerende liposomen. Deze polymeren zijn opgebouwd uit gemodificeerde aminozuren, hetgeen betekent dat ze chemisch gezien overeenkomsten vertonen met eiwitten en dus net als eiwitten biologisch afbreekbaar zijn. Deze polymeren kunnen van belang zijn voor de ontwikkeling van liposomen voor *active drug targeting* (zie pagina 174). De polymeren kunnen bijvoorbeeld bepaalde functionele moleculen in de vetlaag van het liposomen afschermen zolang het liposoom nog niet op de plaats van bestemming is. Na aankomst op de aangetaste plaatsen kan door de verhoogde enzymactiviteit op deze plaatsen de polymeerlaag afgebroken worden zodat de functionele moleculen hun werk kunnen gaan doen.

Zo ver zijn we nu echter nog niet. In dit proefschrift onderzoeken we slechts of *passive drug targeting* mogelijk is. De gevonden resultaten geven aan dat liposomen die bereid worden met de nieuwe polymeren even lang kunnen circuleren als PEG-liposomen met dezelfde afmeting. Ook kunnen op succesvolle wijze glucocorticoïden gestuurd worden naar plaatsen van ontsteking, zoals blijkt uit een studie met deze liposomen in een experimenteel proefdiermodel voor reuma. De resultaten geven aan dat de nieuwe polymeren uitstekende eigenschappen bezitten voor de verdere ontwikkeling van langcirculerende liposomen voor *passive drug targeting*.

4.2 Nieuwe ontwikkelingen op het gebied van de ingesloten glucocorticoïden

Ons huidig onderzoek richt zich niet alleen op de liposomale drager zelf, maar ook op het in te sluiten glucocorticoïde. Op pagina 179 is het belang van het gebruik van glucocorticoïden die in vrije vorm snel door de lever uit de bloedbaan worden verwijderd beschreven. Er zijn echter nog twee andere mogelijkheden om het effect te optimaliseren en de neveneffecten te minimaliseren. Allereerst laten recente wetenschappelijke publicaties een nieuw ontdekt werkingsmechanisme zien waarmee glucocorticoïden ontstekingen kunnen remmen. Het betreft hier een direct effect op celmembranen in plaats van het veelbeschreven remmende effect van glucocorticoïden op de vorming van ontstekingsbevorderende boodschappermoleculen. Dit directe ontstekingsremmende effect staat in het bijzonder op de voorgrond bij bepaalde glucocorticoïden en is alleen van belang wanneer hoge concentraties worden gecreëerd op plaatsen van ontsteking. Wanneer deze glucocorticoïden worden ingesloten in liposomen zou dit werkingsmechanisme dan ook, als gevolg van *targeting* naar plaatsen van ontstekingen, voor een extra ontstekingsremmend effect kunnen zorgen. Deze glucocorticoïden willen we in de nabije toekomst aan nader onderzoek onderwerpen.

Een ander interessant type glucocorticoïden zijn de zogenaamde 11-keto-glucocorticoïden. Deze zijn chemisch zo gemodificeerd dat ze in principe niet actief zijn, maar slechts geactiveerd kunnen worden met behulp van een specifiek enzym (11ß-hydroxysteroïde-dehydrogenase). Aangezien dit enzym zich in relatief hoge concentraties bevindt op plaatsen van ontsteking, zou men door het insluiten van 11-keto-glucocorticoïden in langcirculerende liposomen een extra vorm van selectiviteit kunnen genereren voor de ontstoken plaatsen in het lichaam. Ook deze benadering zal in toekomstig onderzoek aan de orde komen.

In de huidige bereidingswijze wordt uitgegaan van de wateroplosbare natriumfosfaat-vorm van glucocorticoïden waarvan normaal gesproken zo'n 5% van de uitgangshoeveelheid in de liposomen wordt ingesloten als de op pagina 174 beschreven bereidingsmethode wordt gehanteerd. De liposomen die zo gevormd worden kunnen ongeveer een jaar in de koelkast bewaard worden zonder dat er essentiële veranderingen in het preparaat optreden. Om het insluitpercentage van 5% verder te verhogen en de bewaartermijn te verlengen zijn we momenteel bezig met het ontwikkelen van een techniek waarmee voorgevormde, lege liposomen zichzelf kunnen opladen met het aangeboden geneesmiddel. Op deze wijze zou in principe 100% van de uitgangshoeveelheid glucocorticoïde kunnen worden ingesloten. Tevens kunnen liposomen die op deze wijze bereid zijn vaak op eenvoudige wijze gevriesdroogd worden zonder dat dit ten koste gaat van de integriteit van de liposoomstructuur. Vanuit het oogpunt van farmaceutische stabiliteit kunnen gevriesdroogde liposomen veel langer bewaard worden dan liposomen bewaard in de vorm van een waterige dispersie.

5. Liposomaal glucocorticoïde: hoe nu verder?

De meeste studies die in dit proefschrift zijn beschreven zijn preklinische studies, dat wil zeggen: in proefdieren uitgevoerd. Op korte termijn willen we liposomaal glucocorticoïde ook daadwerkelijk in patiënten te testen. Momenteel wordt met de afdeling Reumatologie van het Academisch Ziekenhuis te Nijmegen een planning gemaakt voor een studie bij reumapatiënten. Omdat het doorlopen van de verschillende klinische onderzoeksfasen een zeer kostbaar traject is, wordt overwogen of op termijn commercialisatie van de liposomen haalbaar is. Op die manier kan dan met investeringskapitaal een bedrijf gestart worden dat de liposomen voor de verschillende toepassingen (reuma, MS, vormen van kanker) gaat evalueren in de kliniek. Bij positief resultaat zullen de liposomen voldoende commerciële marktwaarde krijgen om in licentieovereenkomsten verder te worden ontwikkeld door grotere farmaceutische bedrijven. Om deze plannen verder uit te werken is een aanvraag ingediend bij een fonds van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO). Deze aanvraag is inmiddels gedeeltelijk gehonoreerd, in afwachting van nadere evaluaties met betrekking tot de octrooipositie van het op te richten bedrijf.

LIST OF ABBREVIATIONS

AA	adjuvant arthritis
AT-EAE	adoptive transfer-experimental autoimmune encephalomyelitis
BBB	blood-brain-barrier
BOC	tertiary-butoxycarbonyl
BUP	budesonide phosphate
Chol	cholesterol
CIA	collagen type II-induced arthritis
CNS	central nervous system
CO	cardiac output
CVP	central venous pressure
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DODA	dioctadecyl amine
DODASuc	N-succinyl-dioctadecylamine
DPPC	dipalmitoyl phosphatidylcholine
DPTS	4-(dimethylamino)pyridinium-4-toluene sulfonate
DSPC	distearoyl phosphatidylcholine
DSPE	distearoyl phosphatidyl ethanolamine
DTPA	diethylene triaminepenta-acetic acid
DXP	dexamethasone phosphate
EAE	experimental autoimmune encephalomyelitis
EPC	egg phosphatidylcholine
EPG	egg phosphatidylglycerol
GC	glucocorticoid(s)
^{3}H	(radioactive) tritium
HEPES	N-(-2 hydroxyethyl) piperazine-N'-ethane sulfonic acid
HOA	heptadecyl octadecyl amine
i.v.	intravenous(ly)
ID	injected dose
IL-1	interleukin-1
IL-6	interleukin-6
^{111}In	(radioactive) indium-111 isotope
LCL	long-circulating liposome(s)
LEVDP	left ventricular end-diastolic pressure
Mφ	macrophage(s)
MBP	myelin basic protein
MP	methylprednisolone
MPS	mononuclear phagocyte system
MS	multiple sclerosis

MS	multiple sclerosis
NCA	N-carboxy anhydride
ODA	octadecyl amine
PAP	pulmonary artery pressure
PBLA	poly(benzyl L-aspartate)
PBLG	poly(benzyl L-glutamate)
PBS	phosphate buffered saline
PEG	poly(ethylene glycol)
PEG-DSPE	PEG2000-distearoyl phosphatidylethanolamine
PEG-PE	PEG2000-phosphatidylethanolamine
PGPG	poly(hydroxypropyl L-glutamine)
PHBG	poly(hydroxybutyl L-glutamine)
PHEA	poly(hydroxyethyl L-asparagine)
PHEG	poly(hydroxyethyl L-glutamine)
PL	prednisolone liposomes
PL	prednisolone liposomes
PLP	prednisolone phosphate
PVR	pulmonary vascular resistance
RA	rheumatoid arthritis
s.c.	subcutaneous(ly)
SAP	systemic arterial pressure
SC5-b9	protein S (vibronectin)-bound complement terminal complex
SCL	short-circulating liposome(s)
SD	standard deviation
SEM	standard error of the mean
SSL	sterically stabilized liposome(s)
SVR	systemic vascular resistance
TC	T cell(s)
^{99m}Tc	(radioactive) technetium-99m isotope
THF	tetrahydrofurane
TNF-α	tumor necrosis factor-α.

CURRICULUM VITAE

Bart Metselaar was born on July 6th 1971 in Rotterdam, The Netherlands. In 1990 he finished high school education (Dutch system: VWO) at the Melanchton College in Rotterdam and started with the study Pharmaceutical Sciences at the Faculty of Pharmacy, Utrecht University. In 1994 he participated in an undergraduate research program entitled 'Pharmacokinetics and pharmacodynamics of topical glucocorticoids in dogs' at the Department of Pharmaceutics, University of Florida in the USA. He graduated in 1995 and obtained his pharmacist's license in 1997. From 1998 until 2001, Mr. Metselaar has been working as a graduate student at the Department of Pharmaceutics, Utrecht University on his thesis: 'Liposomal targeting of glucocorticoids, a novel treatment approach for inflammatory disorders' under supervision of Prof. Dr. G. Storm, Prof. Dr. D.J.A. Crommelin and Dr. M.H.M. Wauben. Presently he is investigating the possibilities of commercializing the formulations designed and evaluated during this thesis. He aims at setting up a small life science company and successfully applied for financial support from the Dutch organization of Scientific Research ('NWO'), which enables him to further realize this aim. Mr. Metselaar's main research interests are: advanced drug carrier design and optimization, pharmacokinetics and biodistribution of colloidal therapeutics, and immunology and pharmacology of autoimmune disorders. Currently, he lives in Amsterdam and besides enjoying the cultural life and heritage of the Dutch capital city, he spends his free time on culinary activities and (playing) music. He also has a great interest in architecture, astronomy, cartography and geography, philosophy and theology.

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ACKNOWLEDGEMENTS

This thesis would never have been possible without the help and support of so many of you around me: colleagues, friends (often you are both) and family. As i would like to thank each of you in a personal way, i will use my own illegible handwriting on these last two pages of my thesis. (This also gives me hope that anyone else who thinks i owe him/her, be able to recognize his/her name somewhere in this scrawl.) To make finding your name easier, i'll sort everybody in each heading alphabetically.

Let me first address the persons who played a starring role during the realization of this thesis and all the work described in it:

Gert Storm: Gert, als begeleider, expert, net-werker, collega, maar bovenal als persoon heb je heel veel betekend voor mij in de afgelopen jaren. Veel lof!

Marca Wauben: Marca, heel veel is tot stand gekomen dankzij jouw tomeloze inzet, je aanstekelijk enthousiasme en je niet te evenaren wetenschappelijk inzicht.

Daan Crommelin: Daan, je belangstelling en je kritische geest waren van grote waarde voor ons onderzoek.

Strong input also came from the following persons, who did a tremendous job as co-worker, author, co-author, or senior. I learned so much ... thanks!

Wim Hennink, Enrico Mastrobattista, Ingrid Molema, and Raymond Schiffelers

Our 'Nijmegen Connection': Otto Boerman, Peter Laverman and last but not least Peter van Lent

Our 'Würzburg Connection': Ralf Gold, Klaus Toyka, and especially Jens Schmidt

Our 'Washington Connection': Janos Szebeni.

A lot of invaluable, hard work for this thesis was done by:

Josee Wagenaar: Josee, jouw betrokkenheid en gedrevenheid zijn zeldzaam. Dankje wel!

Louis van Blooïs: Louis, de vele moeizame bereidingen werden mogelijk dankzij jouw inzet!

And also by:

Leo de Boer, Peter Bruin, Marcel Fens, Astrid Holthuys.en, Adriënne Jonssen, Marian ten Kate, Richard van Lamoen, Stefan Sanders, and Cor Snel: Heel veel dank voor alle syntheses, de uitgebreide microscopie en de vele in-vitro en in-vivo experimenten!

I also want to thank my congenial UNYPHAR co-workers for their support:
Elmieke Boot, Willem van Eden, Linda Everse, and Gerben Koning.

Special thanks go to my colleagues for their contribution and scientific advise:
Wim Jiskoot, Christien Oussoren, and Mies van Steenbergen. Mies, aan jou heel veel dank voor je hulp bij HPLC- en computergerelateerde problemen.

I am very thankful to my coworkers from Yamanouchi Europe: Peter van Brummelen, Leo de Leede, Kei Motonaga, Margreet Olthoff, Tom de Vringer, and Anneniek van Rensen: thanks for the pleasant and stimulating collaboration over the past years!

I am also greatly indebted to our friendly colleagues from Yamanouchi Japan, among others:
Seiya Kagatani, Yasushi Kawauchi, Akira Takagi, Dr. Yokohama, and Tatsunobu Yoshioka. Thanks for your support and your great hospitality: our stay in Japan will forever be remembered!

In addition, i would like to thank our Polish co-workers: Rafal Hrynik and Marek Langner for the inspiring collaboration and warmly welcoming me in Wroclaw and Krakow.

I would like to mention the students whom i advised in the past four years:
Evy Corbanie, Lia Dekker, Sai Hong Lam, Peter Stöpfer, and Marvin Tjioe
Thanks for your contribution!

and my dear friends outside the scientific world, who have made scientific work so much easier:
Anand, Anissa, Anna, Bart-Jan, Carmen, Cécile, Chejo, Claude, David, Edwine, Fiona, Gijs, Hedi, Ilse, Jenny, Johan, Joost, Judith, Katjusa, Lennart, Liesbeth, Maaike, Marieke, Marieke, Merijn, Mezen, Michiel, Michiel, Mohamed, Muriël, Pamela, Philippine, Pieterd, Rensky, Rien, Rudmer, Ruth, Selina, Sigrid, Suzanne, Waja, and Wendy: Dank jullie wel!

Special thanks to my two 'best men': Marco D'Agata and Willem Vreeken!

Love to my beloved family:
Erik, Helen, John, Leontien, Maarten, Mick, Paulien, Rein en in het bijzonder: Mam en Pap, heel veel dank voor jullie steun.

My valentine, however, deserves most of my attention:
Ingrid, Ingel, Ingbeer: de afgelopen zeven jaar met jou waren de beste tot nu toe...